Development of molecular tools for the rapid assessment of benzimidazole resistance and investigation of possible risk factors in resistance development in *Nematodirus battus* populations

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

Lynsey Anne Melville

May 2019

Development of molecular tools for the rapid assessment of benzimidazole resistance and investigation of possible risk factors in resistance development in *Nematodirus battus* populations

Lynsey Melville

Nematodirus battus is an economically important gastrointestinal nematode (GIN) which threatens lamb health and sustainability of UK farming. In recent years apparent changes in the hatching and infection dynamics have occurred in conjunction with the first reports of fenbendazole resistance The aims of this project were to develop molecular tools to study the BZ-resistant allele frequency in UK *N. battus* populations; examine the hatching preferences (with or without a chill stimulus) of UK *N. battus* populations; assess farmers' perceptions of *N. battus* infection and to gather farm management data to assess current control methods. Finally, to develop generalised linear mixed effect models (GLMM) to investigate possible associations between management decisions and the development of anthelmintic resistance (AR) and the ability of eggs to hatch without chilling.

Three DNA-based detection methods were evaluated within the current project; pyrosequencing, deep-amplicon sequencing by Illumina MiSeq and Loop-mediated isothermal amplification (LAMP) to detect and quantify the single nucleotide polymorphisms (SNPs) within the β-tubulin isotype 1 gene associated with BZ-resistance; namely F176Y, E198A and F200Y. Pyrosequencing and deep amplicon sequencing assays were developed, evaluated and used to conduct a genotyping survey of *N. battus* populations from UK commercial farms. Results were comparable between the two platforms, indicating that either method would be suitable as a laboratory diagnostic tool. LAMP was also evaluated within the project with a view to on-farm diagnosis. The prototype LAMP assay successfully identified the resistant allele when tested using plasmid DNA and was capable of producing

semi-quantitative results. However, further evaluation of the prototype assay using field samples produced inconsistent results.

A total of 282 *N. battus* populations were analysed in the genotyping survey. The F200Y resistant allele was identified in 26% of the populations tested, albeit at low overall allele frequency (~2%) with a focal region of high resistant allele frequency identified in North West England. The F167Y mutation was identified for the first time in *N. battus*, at low prevalence and frequency and no mutations were identified at codon 198. Generalised linear mixed effect model analysis investigating the drivers of BZ-resistance in *N. battus* highlighted set stocked grazing, reseeding of heavily contaminated pasture and the observation of symptoms predominantly in spring as potential risk factors with the administration of quarantine practice as a protective factor.

The farm management questionnaire highlighted significant regional variation in the observation of *N. battus*. The perception of increasing severity of symptoms were reported from respondents in the North whilst the time at which *N. battus* is becoming more apparent to respondents has changed in the South. Diagnostic methods also varied regionally, with greater uptake of faecal egg counting and online risk maps to determine anthelmintic treatments in the South.

The requirement for chilling of *N. battus* eggs prior to hatching was tested in 90 UK *N. battus* populations *in vitro.* Up to 87% of eggs were able to hatch without chilling, with higher non-chill hatching observed in Scottish populations. GLMM analysis, identified associations between spring temperature, lambing date, *N. battus* faecal egg count and grazing management strategies with non-chill hatching. The results indicated that the requirement for chilling may vary throughout the year, possibly in response to host immune development.

This project represents the largest survey of UK *N. battus* populations ever conducted and has provided a valuable insight into how this species has changed in recent years. Epidemiological differences observed North to South questions the suitability of the current 'one fits all' approach to advice. Validation of the risk factors identified, could provide the basis for novel control strategies to minimise production losses and the associated economic cost of *N. battus* infection.

Acknowledgements

Firstly, I'd like to thank my supervisor Dave Bartley for his support throughout the project. For taking the time to listen to my thoughts and ideas from the impact of Chernobyl nuclear fall-out on nematode populations to following migratory geese and wild deer. I really appreciate all the time, effort and encouragement over the past four years. Thank you also to my supervisors Jan van Dijk and Sian Mitchell for your continued support and advice and to Giles Innocent for your endless patience whilst teaching me to use R and to embrace statistics and modelling!

A huge thanks to all of the Moredun regional advisors, vets and farmers who enthusiastically supported this project. Particularly the farmers of Longsleddale valley, Judith Lees and Ian Richards, without whom this project would not have been possible.

Thanks to the whole parasitology team at Moredun for your help in the lab and for keeping me laughing no matter how many samples arrived. Particularly Alison Morrison for getting me started with the project and for helping me collect samples come rain or shine, it was very much appreciated! A special thanks also to Fiona Sargison and Catriona Aitkin for their help in tackling my mountain of samples over the summer.

I would also like to thank John Gilleard for allowing me to visit his lab in the University of Calgary during the third year of my project to learn about deep amplicon sequencing. Thanks to Libby Redman and Russel Avramenko for their help in the lab and for making me feel so welcome during my visit.

I am grateful for the funding I received from the Animal and Horticultural Development Board to conduct this project and to Liz Genever for overseeing my progress and for helping to recruit AHDB members to take part in the project. Thanks also to MAST Ltd., particularly Seamus Stack for help with the design and troubleshooting of my LAMP assay and for providing reagents during the development and evaluation of the test.

Contents

Acknowledgements 5
List of Figures 11
List of Tables 13
List of abbreviations 15
1 Introduction 17
1.1 Parasitology and lifecycle 17
1.2 Pathology 20
1.3 Immune development in exposed lambs 24
1.4 Historic and current prevalence 26
1.5 Epidemiology 28
1.6 Predictive models of *N. battus* risk 32
1.6.1 Risk factor analysis 33
1.7 Control strategies 34
1.7.1 Management 34
1.7.2 Nutrition 36
1.7.3 Chemical 37
1.7.4 Benzimidazole-resistant *N. battus* 41
1.7.5 Origins of benzimidazole resistance 42
1.7.6 Drivers of benzimidazole resistance 43
1.7.7 Detection of anthelmintic resistance 48
1.8 Prevalence of BZ-resistance 59
1.8.1 Trichostrongylid species 59
1.8.2 *Nematodirus battus* 62
1.9 Aims of the project: 63
2 Prevalence of SNPs associated with benzimidazole resistance in UK *N. battus* populations 65
2.1 Abstract 65
2.2 Introduction 66
2.3 Methods 67
2.3.1 Sample collection 67
2.3.2 Sample preparation and DNA extraction 69
2.3.3 DNA amplification and pyrosequencing 71
2.3.4 Statistical analysis 72

2.4 Results 74
2.4.1 Faecal egg counts 74
2.4.2 Prevalence of F167Y in UK *N. battus* populations 77
2.4.3 Prevalence of E198A in UK *N. battus* populations 79
2.4.4 Prevalence of F200Y in UK *N. battus* populations 79
2.4.5 Analysis of genotype profiles over time: repeated samples 82
2.4.6 Mutual exclusivity of F200Y and F167Y 84
2.4.7 Hardy-Weinberg analysis 86
2.5 Discussion 86
2.6 Conclusions 91
3 Development and evaluation of a loop-mediated isothermal amplification assay for the sensitive detection and quantification of single nucleotide polymorphisms associated with benzimidazole resistance in *Nematodirus battus* 92
3.1 Abstract 92
3.2 Introduction 93
3.3 Methods 97
3.3.1 Preparation of gDNA standards 97
3.3.2 Preparation of plasmid DNA standards 98
3.3.3 Preparation of individual and pooled field samples 99
3.3.4 Pooled *N. battus* L_3 extracts 99
3.3.5 Sequencing of the β-tubulin isotype 1 gene in *N. battus* 100
3.3.6 Workflow of design and evaluation 101
3.3.7 Primer design 103
3.3.8 LAMP reaction set up 105
3.3.8.1 Lyophilised V6.21 pellet reagents 105
3.4 Results 108
3.4.1 β-tubulin isotype 1 gene sequence 108
3.4.2 Initial testing of primer sets 108
3.4.3 Repeatability testing 112
3.4.4 Optimisation 115
3.4.5 Quantification 118
3.4.6 Application of assay using field samples 121
3.5 Discussion 124
3.6 Conclusion 130

4 Comparison of next generation deep amplicon sequencing and pyrosequencing technologies in the detection and quantification of benzimidazole resistance in UK *N. battus* populations 132
4.1 Abstract 132
4.2 Introduction 133
4.3 Materials and Methods 134
4.3.1 Parasite material and DNA extraction 134
4.3.2 Amplification of the target sequencing region and preparation of the sample library for sequencing 135
4.3.3 Analysis pipeline for Illumina sequence data 137
4.3.4 Statistical Analysis 138
4.4 Results 139
4.4.1 Prevalence of F167Y in UK *N. battus* populations 139
4.4.2 Prevalence of E198A in UK *N. battus* populations 139
4.4.3 Prevalence of F200Y in UK *N. battus* populations 140
4.4.4 Comparison of MiSeq and pyrosequencing results 142
4.5 Discussion 147
4.6 Conclusions 152
5 Descriptive analysis of UK farm management practices and current *N. battus* control strategies 154
5.1 Abstract 154
5.2 Introduction 155
5.3 Materials and Methods 156
5.3.1 Interviews 156
5.3.2 Questionnaire design 156
5.3.3 Question selection 157
5.3.4 Piloting 158
5.3.5 Dissemination 158
5.3.6 Analysis 158
5.4 Results 159
5.4.1 Farm demographics 159
5.4.2 Perception of N. battus on farm 164
5.4.3 Timing of infection 165
5.4.4 Changes in *N. battus* infection on farm in recent years 169
5.4.5 Grazing management 171

5.4.6 Anthelmintic control 172
5.4.7 Quarantine 178
5.5 Discussion 181
5.6 Conclusions 190
6 Drivers and barriers for the selection of benzimidazole resistance in *Nematodirus battus* 191
6.1 Abstract 191
6.2 Introduction 192
6.3 Methods 194
6.3.1 Data Sources 194
6.3.2 Generalised linear mixed model screening analysis and model fitting 195
6.4 Results 199
6.4.1 Screening analysis 201
6.4.2 Fitted multivariable models 209
6.4.3 Model Selection 215
6.5 Discussion 216
6.6 Conclusion 226
7 *Nematodirus battus* egg hatching: Investigating the drivers of change 227
7.1 Abstract 227
7.2 Introduction 228
7.3 Methods 231
7.3.1 Sample collection 231
7.3.2 Sample preparation 233
7.3.3 Hatching experiments 234
7.3.4 Model construction 234
7.4 Results 237
7.4.1 Comparison of F200Y resistant allele frequency and altered hatching requirements in *N. battus* populations 239
7.4.2 Investigation of drivers 240
7.5 Discussion 252
7.5.1 Impact of environmental factors 256
7.5.2 Impact of management factors 257
7.5.3 Impact of host factors 259
7.6 Conclusion 263

8 General discussion 264
Appendix 1 275
Appendix 2 276
Appendix 3 278
Appendix 4 280
Appendix 5 294
Appendix 6 295
9 References 310

List of Figures

Chapter 1 Figures

Figure 1.1. Typical N. battus lifecycle. *17*
Figure 1.2. Infective Nematodirus battus larvae hatching from their eggs. *20*
Figure 1.3. Lamb with breech soiling, a clinical sign of N. battus infection. *21*
Figure 1.4. Scanning electron micrographs of intestinal damage caused by N. battus. *22*
Figure 1.5. Parasite surveillance data from the Animal and Plant Health Agency. *30*
Figure 1.6. Diagram of the mode of action of benzimidazole. *39*
Figure 1.7. Overview of pyrosequencing methodology. *52*
Figure 1.8. Illumina MiSeq next generation sequencing method overview. *54*
Figure 1.9. Diagram of the amplification method of Loop-mediated isothermal amplification (LAMP). *58*
Figure 1.10. Timeline of benzimidazole resistance in ovine nematode species. *61*

Chapter 2 Figures

Figure 2.1. Flow diagram of how many farm samples were collected from surveillance centres, AHDB and Moredun members and in-person farm visits during each sampling year *68*
Figure 2.2. N. battus eggs at different development stages. *70*
Figure 2.3. Map of UK regional boundaries used to divide sample populations for data analysis throughout the project. *73*
Figure 2.4. Nematodirus faecal egg count results. *75*
Figure 2.5. Strongyle faecal egg count results. *76*
Figure 2.6. Boxplot of individual N. battus faecal egg count per UK region. *77*
Figure 2.7. Map of the distribution of N. battus isolates in which the F167Y allele was identified. *78*
Figure 2.8. Map of the distribution of N. battus isolates in which the F200Y allele was identified. *81*
Figure 2.9. F200Y resistant allele frequency of repeated samples. *82*
Figure 2.10. F200Y genotype frequencies obtained from repeated sampling. *83*
Figure 2.11. Genotype profile (% homozygous susceptible (SS), heterozygous (Sr) and homozygous resistant (rr)) of the F200Y and F167Y codons of the 19 farms analysed at both loci by pyrosequencing *85*

Chapter 3 Figures

Figure 3.1. Application of loop-mediated isothermal amplification in a point-of-care device. *95*
Figure 3.2. Work flow of LAMP assay design and evaluation *102*
Figure 3.3. Sub-section of the DNA sequence of the N. battus β-tubulin gene with LAMP primers highlighted. *105*
Figure 3.4. Progress chart of LAMP primer sets. *109*
Figure 3.5. Amplification plots of initial tests conducted using the BIP3 primer set. *111*
Figure 3.6. Repeatability tests using BIP1 primer set. *113*
Figure 3.7. Repeated LAMP reactions using BIP3 primer set. *114*
Figure 3.8. Prototype F200Y LAMP assay amplifying plasmid DNA. *115*
Figure 3.9. Quantification test of prototype LAMP assay *119*
Figure 3.10. Quantification test of prototype LAMP assay using plasmid DNA. *120*
Figure 3.11. Test of prototype LAMP assay with DNA extracted from field samples. *121*

Figure 3.12. Test of prototype LAMP assay with DNA extracted from pooled eggs/L_3. ..... *122*
Figure 3.13. Test of prototype LAMP assay with DNA extracted from pools of 100 L_3 ..... *123*

Chapter 4 Figures

Figure 4.1 Comparison of the results of F200Y analysis between pyrosequencing and MiSeq regarding the presence or absence of resistant alleles and the range of resistant allele frequencies observed. *143*
Figure 4.2 Mean F200Y allele frequency by UK region using pyrosequencing and MiSeq analysis.... *144*
Figure 4.3. Bland Altman comparison of F200Y allele frequency results obtained from pyrosequencing and next generation amplicon sequencing *146*

Chapter 5 Figures

Figure 5.1. Map of questionnaire respondents *160*
Figure 5.2. Histogram plots of the timing of peak lambing on farms split by geographic region. *162*
Figure 5.3. Map of main sheep breed kept by questionnaire respondents. *163*
Figure 5.4. Summary of the season in which respondents typically observed symptoms of N. battus. *166*
Figure 5.5. Animal and Plant Health Agency N. battus surveillance data for England January 2015/December 2017..... *168*
Figure 5.6. Summary of the changes in N. battus infection reported by respondents. *170*
Figure 5.7. Comparison of parasite monitoring method and the typical number of anthelmintic treatments administered annually to lambs *177*
Figure 5.8. Quarantine practice employed by UK region..... *179*
Figure 5.9. Predicted date of first N. battus larvae available on pasture each year *187*

Chapter 6 Figures

Figure 6.1. Observation of N. battus symptoms on farms with and without F200Y resistant alleles present. *204*
Figure 6.2. Quarantine practice on farms with varying F200Y resistant allele frequency..... *205*
Figure 6.3. Quarantine isolation time on farms with varying F200Y resistant allele frequency..... *206*
Figure 6.4. Anthelmintic class used for quarantine treatment on farms with varying F200Y resistant allele frequency *207*
Figure 6.5. Causal web analysis examining the biological interaction between risk factors tested within the current GLMM model..... *209*

Chapter 7 Figures

Figure 7.1. Venn diagram of factors currently believed to impact on the hatching of N. battus eggs following a chill stimulus from literature *230*
Figure 7.2. Map of the origins of N. battus populations included in the hatching study *232*
Figure 7.3. N. battus egg at the embryonated stage, L_3 visible inside the egg *233*
Figure 7.4. Summary of chill requirement of eggs from Scotland and England/Wales..... *238*
Figure 7.5. Comparison of F200Y resistant allele frequency and chill requirement of egg hatching . *239*
Figure 7.6. Comparison of lamb age at sampling and the requirement for chilling in N. battus egg hatching. *247*
Figure 7.7. Predictions from model 3 ..... *251*
Figure 7.8. Factors highlighted by the current analysis as having a significant impact on the hatching of N. Battus eggs in vitro..... *255*

List of Tables

Chapter 1 Tables

Table 1.1. Anthelmintic compounds currently licensed for use in the UK, the year each was introduced to the market and the parasites targeted by each class 37
Table 1.2. Farm management, parasite and host factors previously associated with the development of anthelmintic resistance in sheep nematodes 47
Table 1.3. Summary table of the five major anthelmintic classes. 60

Chapter 2 Tables

Table 2.1. PCR and sequencing primers for genotyping the F167Y SNP in N. battus by pyrosequencing 72
Table 2.2. Mean regional F200Y allele frequency identified by pyrosequencing in Scottish, English and Welsh N. battus farm populations. 79

Chapter 3 Tables

Table 3.1. Details of pooled L_3 DNA extractions. 100
Table 3.2. Primer sequences designed during the LAMP development study. 104
Table 3.3. Inner (FIP/BIP) and outer (F3/B3) primer concentrations included in primer ratio variation. 107
Table 3.4. Results of initial tests using each primer set 110
Table 3.5. Summary of LAMP optimisation tests 116

Chapter 4 Tables

Table 4.1. N. battus-specific PCR primers used to amplify the β-tubulin isotype 1 gene exon 4-5..... 135
Table 4.2 Mean farm genotyping results from the analysis of codon 167 by pyrosequencing and MiSeq 139
Table 4.3 Mean regional F200Y allele frequency identified by pyrosequencing (pyro) and Illumina MiSeq in Scottish, English and Welsh N. battus farm populations 141

Chapter 5 Tables

Table 5.1. Summary of anthelmintic classes used to control N. battus and other nematode species 173
Table 5.2. Summary of parasite monitoring methods used to determine the timing of anthelmintic treatment. 174
Table 5.3. Regional use of faecal egg counting and yearly prophylactic treatments in the control of GIN. 176

Chapter 6 Tables

Table 6.1. A list of the covariates included in GLMM analysis including the units of each factor and the coding method used (categorical, continuous or binary)..... 197
Table 6.2. Summary of the farm demographics of farms included in the genotyping study..... 200
Table 6.3. Results of univariate screening GLMM analysis. 202
Table 6.4. Model 1. Results of GLMM (fitted with a logit link) analysis with binomial outcome of the number of F200Y resistant alleles identified out of the total number of alleles identified on each farm. Results are presented as odds ratios, 95% confidence intervals and p-values (Wald tests) 211
Table 6.5. Model 2. Results of GLMM (fitted with a logit link) analysis with binomial outcome of the number of F200Y resistant alleles identified out of the total number of alleles identified on each farm. Results are presented as odds ratios, 95% confidence intervals and p-values (Wald tests). 212
Table 6.6. Model 3. Results of GLMM (fitted with a logit link) analysis with binomial outcome of the number of F200Y resistant alleles identified out of the total number of alleles identified on each farm. Results are presented as odds ratios, 95% confidence intervals and p-values (Wald tests). 213
Table 6.7. Model 4. Results of GLMM (fitted with a logit link) analysis with binomial outcome of the number of F200Y resistant alleles identified out of the total number of alleles identified on each farm. Results are presented as odds ratios, 95% confidence intervals and p-values (Wald tests). 214

Chapter 7 Tables

Table 7.1. Model 1. Results of GLMM analysis with binomial outcome of hatching success in N. battus eggs (fitted with a logit link)). Covariates included were whether or not a chill stimulus was applied and a number of climatic variables; minimum/maximum temperature in spring/autumn, precipitation, number of hours of sun, farm elevation, farm position and experimental factors. Results are presented as odds ratios with 95% confidence intervals and p-values (Wald tests) 241
Table 7.2. Model 2. Results of GLMM analysis with binomial outcome of hatching success in N. battus eggs (fitted with a logit link). Covariates included were whether or not a chill stimulus was applied and a number of farm management practices; away grazing, grazing strategy of lambs, resting and reseeding highly contaminated fields, peak lambing week, faecal egg count of the faecal samples from which eggs were extracted and experimental factors. Results are presented as odds ratios with 95% confidence intervals and p-values (Wald tests)..... 244
Table 7.3. Model 3. Results of GLMM analysis with binomial outcome of hatching success in N. battus eggs (fitted with a logit link). Covariates included were whether or not a chill stimulus was applied, peak lambing week, farm type and experimental factors. Results are presented as odds ratios with 95% confidence intervals and p-values (Wald tests)..... 250

Chapter 8 Tables

Table 8.1. Potential levels of refugia for N. battus predicted from a number of climatic, management and hatching scenarios 270
Table 8.2. Summary of the key findings of the project and research ideas to further explore and/or implement industry recommendations based on the findings. 273

List of abbreviations

Abbreviation	Meaning
AD	Monepantel
AHDB	Animal and horticultural development board
APHA	Animal and plant health agency
AR	Anthelmintic resistance
bp	Base pair
BZ	Benzimidazole
CET	Controlled efficacy testing
DEFRA	Department for environment, food and rural affairs
d.f.	Degrees of freedom
DNA	Deoxyribonucleic acid
E198A	Switch from glutamic acid to alanine at codon 198 of the β-tubulin isotype 1 gene
EHA	Egg hatch assay
EID	Electronic identification tag
EPG	Eggs per gram
EtOH	Ethanol
FECRT	Faecal egg count reduction test
F167Y	Switch from phenylalanine to tyrosine at codon 167 of the β-tubulin isotype 1 gene
F200Y	Switch from phenylalanine to tyrosine at codon 200 of the β-tubulin isotype 1 gene
gDNA	Genomic DNA
GIN	Gastrointestinal nematode
GLMM	Generalised linear mixed models
iNAAT	Isothermal nucleic acid amplification technology
KCl	Potassium chloride

L_3		Third stage nematode larvae
LAMP		Loop-mediated isothermal amplification
LV		Levamisole
$MgCl_2$		Magnesium chloride
MgSO4		Magnesium sulphate
ML		Macrocyclic lactones
PBS		Phosphate buffered saline
PCR		Polymerase chain reaction
POC		Point of care
qPCR		Quantitative polymerase chain reaction
qGIS		Quantum geographic information system
SCOPS		Sustainable control of parasites in sheep
SI		Derquantel & abamectin (Startect)
SEM		Standard error of the mean
SNP		Single nucleotide polymorphism
SRUC		Scotland's rural college
T_m		Melting temperature for PCR primers
X^2		Chi-squared test result
Regions	NS	North Scotland
	SS	South Scotland
	NE	North East England
	NW	North West England
	SC	South central England
	SE	South East England
	SW	South West England
	W	Wales

1 Introduction

1.1 Parasitology and lifecycle

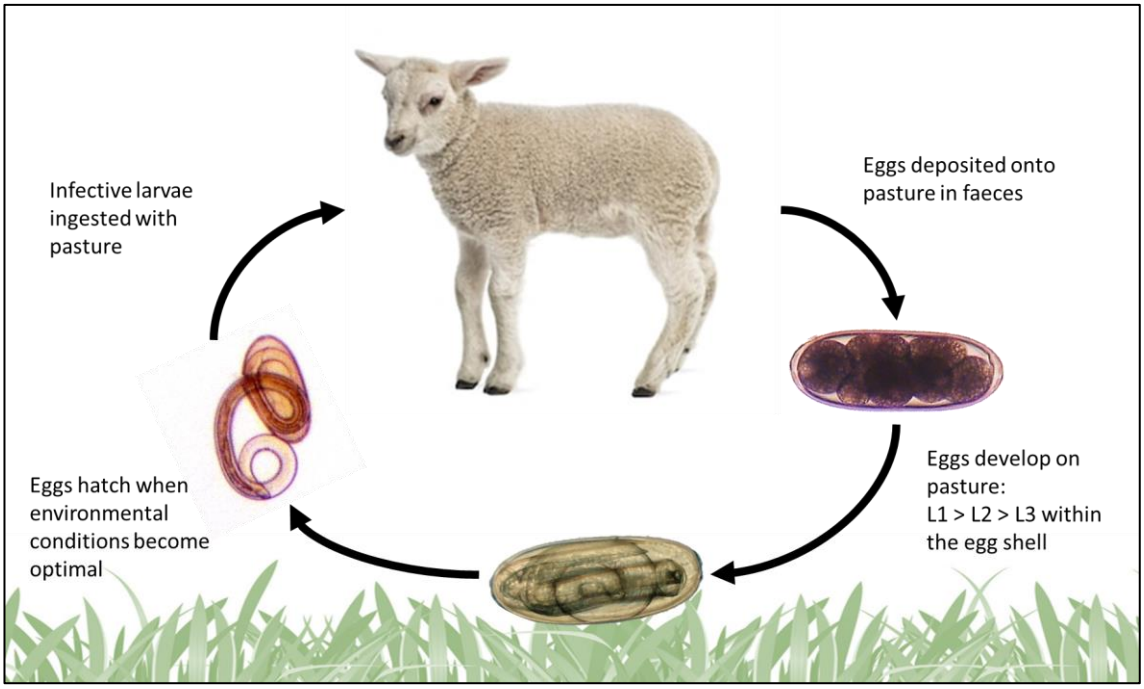


Figure 1.1. Typical N. battus lifecycle.

Eggs passed out onto pasture in faeces, develop to L3 stage within the egg shell. Developed eggs remain intact on pasture until environmental conditions are optimal for hatching then L3 emerge and are ingested by grazing animals.

Nematodirus battus is a gastrointestinal nematode (GIN) that affects both small and large ruminants. It was first described in UK sheep in 1951 (Crofton and Thomas, 1951) and remains a major threat to lamb health throughout the UK and many other temperate regions of the world. *N. battus* is believed to have originated in arctic regions and transferred via animal movements (Hoberg, 2005). This nematode has a direct parasitic lifecycle similar to those of other GIN but with the major difference that the infective larval stage develops within the egg rather than on pasture with the egg-development phase therefore taking place over a much longer period (e.g. months in *N. battus* versus up to a week in other GIN; Figure 1.1. After migration of infective larvae (L_3), onto pasture, they are ingested with

herbage, the larvae exsheath within the abomasum and progress to their predilection site, the small intestine (Mapes and Coop, 1972). Larvae initially burrow deep within the mucosa to continue development, undergoing the fourth moult in this site before re-emerging 5-12 days post infection to complete their development at the surface (Mapes and Coop, 1972; Thomas, 1959a). Progression from an exsheathed L_3 to L_4 to L_5 and finally to sexually mature adult can occur in as little as 14 days post infection (Mapes and Coop, 1972). *N. battus* are dioecious, adult worms mate and females produce eggs which are then excreted in the host faeces. Given a high larval challenge, immunity to *N. battus* infections typically develops rapidly and dramatically, with the majority of adult worms being expelled 24-34 days post-infection after which lambs recover (Mapes et al., 1973; Martin and Lee, 1976). If the larval burden is too high, mortality rates can be significant (Kingsbury, 1953). Severe damage to the small intestine can result in lambs being unable to retain water and thus succumb to dehydration quickly, often before significant number of *N. battus* eggs are present in the faeces.

The development of larvae to L_3 within the egg means that the larvae are protected from adverse environmental conditions, which typically result in low egg mortality and consequently high challenge on pasture. The timing of development and hatching generally depends on climatic conditions. Development occurs within the temperature range of 11.5 - 27˚C and generally takes around 4 weeks (Thomas, 1959a; van Dijk and Morgan, 2008) but hatching does not occur immediately upon completion of development (Boag and Thomas, 1975; Gibson, 1963; Thomas, 1959b). The eggs typically require a period of chilling (below 11°C) prior to hatching, the length of chilling has been shown to influence the proportion of eggs hatching with maximum hatch occurring after a chill of 12 weeks or more (van Dijk and Morgan, 2008), which would be easily reached in nature throughout the winter months. When spring day/night temperature stabilises within the optimum range for *N. battus*; estimated to be between 11 and 17˚C (van Dijk and Morgan, 2008) for around 10 consecutive

days, eggs undergo synchronous mass-hatching, resulting in very high challenge from contaminated pastures.

The exact role of the chill stimulus in *N. battus* hatching remains unclear. Chilling eggs causes an increase in carbohydrate accumulation within the eggs with lipid energy reserves converted into trehalose sugar (Ash and Atkinson, 1983) which has been associated with overcoming thermal stress in nematodes (Jagdale and Grewal, 2003); the increase in trehalose concentration protects the larvae by lowering the freezing point within the egg (Ash and Atkinson, 1983; Jagdale and Grewal, 2003). The requirement for chilling to increase cold-hardiness may be a relic of the arctic origins of this species (Hoberg, 2005) to protect the larvae from extremely low winter temperatures and to prevent eggs hatching in late summer/autumn when suitable hosts may not be grazing. Chilling of eggs has also been linked with increased activity and longevity of the resultant larvae on pasture (van Dijk and Morgan, 2010) which would benefit larvae searching for sparse hosts given arctic conditions however, these adaptations may be less important in UK intensive farming environments.

N. battus egg hatching (Figure 1.2) occurs following the release of hatching fluid from the larvae; a mix of histolytic enzymes including esterase and chitinase which weaken the egg shell (Rogers, 1958). The trigger for the release of hatching fluid is unclear. The egg shell regulates the internal environment, maintaining osmotic restraint, the egg shell becomes more permeable prior to hatching (Ash and Atkinson, 1984), releasing the osmotic pressure within the shell which could be the stimulus for larvae within the egg to release the hatching fluid.

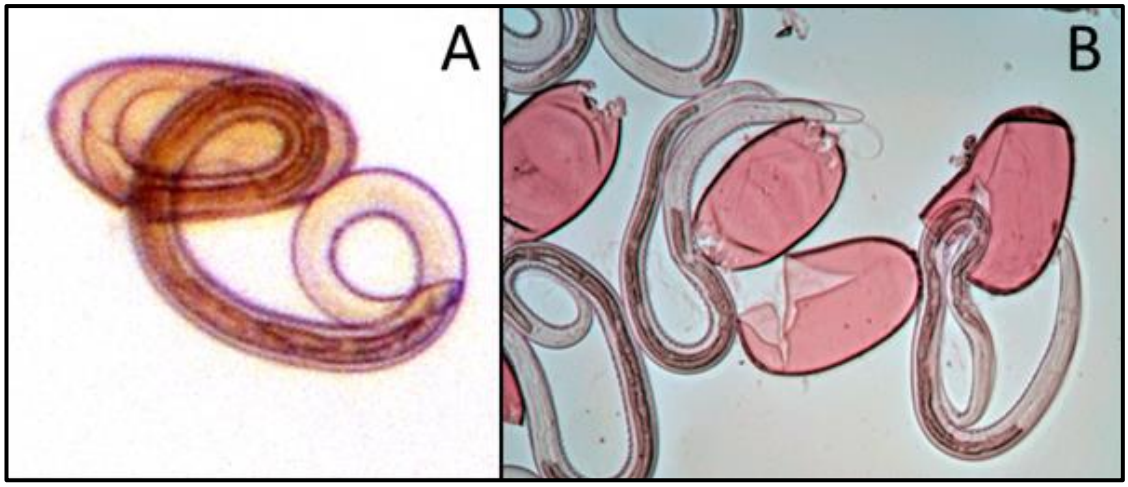


Figure 1.2. Infective Nematodirus battus larvae hatching from their eggs.

The pictures illustrate developed N. battus eggs hatching (a) naturally following a chill incubation and (b) artificial hatching from eggs cracked between glass plates.

Once on pasture, infective larvae have a short lifespan; reduced to 20% of that observed following the peak hatch within one month and almost zero after three months (Thomas and Stevens, 1956; van Dijk et al., 2009). The mortality rate of larvae on pasture has been shown to be governed by environmental conditions, rising with exposure to ultra violet radiation and constant or fluctuating temperatures above 17˚C (van Dijk et al., 2009; van Dijk and Morgan, 2008). The synchronous hatching of this species, high challenge and rapid decay restrict this parasite to the characteristic spring infection of young lambs where clinical symptoms are often severe.

1.2 Pathology

Acute disease, known as *Nematodirosis*, occurs when synchronous hatching of *N. battus* eggs coincides with the grazing of naïve animals, particularly young lambs. Infection is common in lambs around 6-8 weeks of age, causing acute yellow/green diarrhoea and subsequent dehydration (Figure 1.3) (Kingsbury, 1953).

Figure 1.3. Lamb with breech soiling, a clinical sign of N. battus infection.

Symptoms result from damage to the intestinal epithelium during establishment and development of the incoming larvae (Kates and Turner, 1955; Mapes and Coop, 1972). The majority of pathology is caused by juvenile *N. battus* rather than adult parasites as these are more intimately associated with the tissue and move more vigorously than adult nematodes, causing mechanical damage (Kates and Turner, 1955; Mapes and Coop, 1972). Intestinal damage has been shown to build throughout the infection, particularly during the first 12 days (Kates and Turner, 1955; Thomas, 1959b) after which point mature, adult nematodes typically remain on the surface of the intestine (Figure 1.4a) (Mapes and Coop, 1972). The extent of intestinal damage varies and can range from mild, patches of surface erosion to acute inflammation of the organ and in severe cases, erosion or loss of intestinal villi can occur, significantly reducing the activity of the small intestine (Coop et al., 1973; Kingsbury, 1953; Thomas and Stevens, 1956). Changes in the epithelial cells on the surface of intestinal villi have also been reported in some cases where short, narrow brush borders with sparse, distorted microvilli and flattened ridge structures such as that depicted in figure 1.4b reduce the absorptive potential of the cells (Coop et al., 1973).

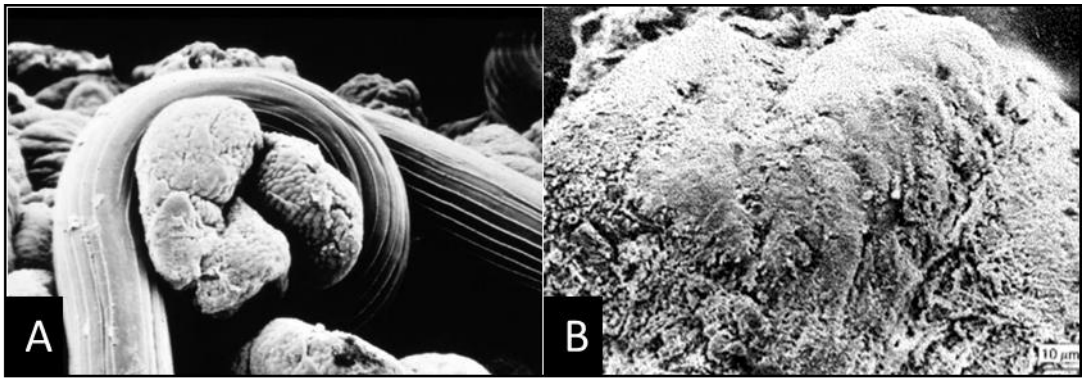


Figure 1.4. Scanning electron micrographs of intestinal damage caused by N. battus.

Scanning electron micrographs of the mucosal surface of the small intestine of a lamb infected with Nematodirus battus (a) Adult N. battus worm coiled around microvilli in the small intestine of a parasitized lamb. [Image courtesy of Moredun Research Institute] (b) Degradation of the small intestinal villi 20 days post infection [Image taken from (Martin and Lee, 1980b) with the permission of Cambridge University Press].

It has been hypothesised that the flattened structure observed in animals given a high larval challenge may be involved in the expulsion of adult worms from the intestine 22-34 days post infection as *N. battus* is believed to use the structure of the villi to retain their position and prevent being washed further down the length of the intestine (Martin and Lee, 1980b). Martin and Lee (1980b) described reversion of the intestinal damage and the changes observed in the structure of the villi following the expulsion of adult *N. battus* 32 days post infection where the structure of the villi appeared to resemble that of uninfected lambs. The majority of *N. battus* (87%) reside within the first 25% of the small intestine (Mapes and Coop, 1972), likely due to the physiochemical conditions which vary significantly along the length of the organ. The physiological conditions at the posterior region of the small intestine are inappropriate for *N. battus* to establish and maintain infection, as a result, few *N. battus* are observed in this region and those which are have been found to grow and develop at a slower rate and have fewer eggs in utero (Mapes and Coop, 1972). Similar retardation of nematode development was observed during co-current infection of *N. battus* with high burdens of *Haemonchus contortus*, attributed to the altered conditions in the small intestine

as a result of changes to the pH and electrolyte concentrations in the abomasum (Mapes and Coop, 1970, 1971, 1973). Factors such as pH and digesta flow rate at the time of infection may be important in determining the resultant worm burden in sheep, perhaps accounting for significant individual variation in parasite numbers and intestinal damage (Mapes and Coop, 1972).

Studies have documented a reduction in food intake by approximately 28% in lambs infected with *N. battus* compared with uninfected animals around the time that juvenile worms typically emerge from the mucosal layer; 8-15 days post-infection (Rowlands and Probert, 1972). The efficiency of nutrient utilisation was also found to be lower in parasitized animals due to mucosal damage in the intestine (Kates and Turner, 1955), coupled with a temporary reduction in weight gain. Despite weight gain recovering later in the season, the check in growth due to *N. battus* at a young age results in a permanent reduction in liveweight compared to uninfected animals (Rowlands and Probert, 1972). As with other helminth infections, *N. battus* has been shown to reduce the activity of several intestinal enzymes including leucine aminopeptidase, alkaline phosphatases and disaccharidase which are involved in protein and sugar digestion, reducing nutrient utilization in infected animals (Coop et al., 1973; Hoste et al., 1993). The exact mechanism by which helminths alter gut enzymes is poorly understood. Surface damage, such as the patches of denudation at the tips of microvilli and damage to the epithelial brush border observed following heavy *N. battus* challenge may be implemented in altering the pH of the organ and therefore interfering with enzyme activity or production (Coop et al., 1973; Rowlands and Probert, 1972). Characteristic scouring in lambs results from the intestinal damage caused by juvenile *N. battus* and the hypersensitivity immune response mounted by the host. Increased recruitment of eosinophils, triggering of a Th2 response and muscle hypercontractility have also been associated with nematode expulsion (Khan and Collins, 2004; Larsen et al., 1999; Marzio et al., 1990).

The intensity of the infection varies between lambs; the majority will recover within a month, however mortality rates for *N. battus* infection can be as high as 10-30% of the lamb crop in which animals suffer intense scouring and succumb to dehydration quickly (Kingsbury, 1953). Other clinical signs include sunken eyes and rough wool, lambs have been observed to have a 'tucked up' appearance, signalling abdominal pain and an unwillingness to move unless forced (Gibson and Everett, 1973; Kingsbury, 1953; Stamp and Dunn, 1955).

1.3 Immune development in exposed lambs

It has been well documented that lambs are capable of mounting a protective immune response to *N. battus* from a young age, which is typically maintained throughout life (Israf et al., 1997; Taylor and Thomas, 1986). The acquired immune response mounted by young lambs is believed to be responsible for the rapid expulsion of adult worms following heavy challenge (Mapes and Coop, 1972). Aquired immunity also provides protection against re-infection, causing retardation of adult worms and significantly lower establishment of incoming L_3 (Taylor and Thomas, 1986). The developmental retardation of adult worms in 'immune' animals is similar to that described in response to changes in the physiological conditions of the small intestine as a result of concurrent infection with *N. battus* and *H. contortus* described above (Mapes and Coop, 1970, 1971), suggesting that the parasite responds in a similar fashion to a variety of stimuli and stressors. Adult sheep previously exposed to *N. battus* can be re-infected but egg output is generally low with few clinical signs observed (Thomas, 1959b). However, infection in adult sheep which did not develop sufficient immunity as lambs can cause *Nematodirosis* and losses have been increasingly reported in recent years (Sargison et al., 2012).

Development of the acquired immune response has been shown to be dependent upon both the size of the initial parasitic challenge and the individual immunological response of the animal (Taylor and Thomas, 1986). Previous studies have noted individual variation in

immune response mounted by lambs based on adult worm burden and the length and developmental stage of the parasites recovered post mortem following experimental challenge (Israf et al., 1996). Following a large challenge lambs typically mount a hypersensitivity reaction resulting in the expulsion of a large proportion of the established adult worm population within 21 days post infection (Winter et al., 1996). Lambs have been shown to produce a specific IgM and IgG antibody response from around 18 days post infection (Winter et al., 1996) and a significant increase in the number of eosinophils and mast cells in the region of the intestine where adult worms have established (Winter et al., 1997b). The Th2-type response is believed to be responsible for the rapid reduction in adult worm burden. Although a small number of adult worms typically remain following the reduction in cellular immune response around 25-28 days post infection (Winter et al., 1997b). Examination of the small intestine of the host by scanning electron microscopy around the time of adult worm expulsion showed the nematodes encased in tubes of mucous-based material which were hypothesised to be involved in structural changes observed within the nematode and ultimately, their removal from the small intestine (Martin and Lee, 1980b). Close analysis of adult worms collected 22-34 days post infection following a high level infection showed the formation of crystals within the lumen of the intestine of the worms (Lee and Martin, 1980). The sulphur-rich lipoprotein crystals were found in increasing number and size from day 20 post-challenge onwards. The crystals were believed to have formed within the worms and were progressively larger from the mid to posterior end of the intestinal tract where they blocked the intestinal-rectal junction causing a build-up of fluid behind the blockage (Martin and Lee, 1975). Formation of the crystals was believed to have been associated with the development of immunity in infected lambs as no crystals were found in adult worms recovered from lambs infected with low numbers of *N. battus* and which exhibited little or no immune response. Additional changes observed

include the disorganisation and apparent degradation of the structure of the reproductive organs of both male and female nematodes (Martin and Lee, 1980a).

1.4 Historic and current prevalence

N. battus was first described in the Scottish borders in 1951 (Crofton and Thomas, 1951) and quickly became endemic throughout the UK however, the way in which this species was introduced to the British Isles remains a mystery. Some authors have hypothesised that the species was present in the UK for a long time but increased significantly following the intensification of farming in the 1940s (Zayed, 2016). Alternatively, the sudden appearance of *N. battus* in the 1950's may indicate a more recent introduction. Perhaps with the importation of non-native deer species (Jansen, 1973) or migratory geese which are often observed grazing in livestock fields (Hollands, 2018), either through ingestion and shedding of eggs or by physical transfer of contaminated sheep faeces on their feet.

A phylogenetic study of geographically isolated *N. battus* populations identified minimal differences between sequences from isolates collected from Norway and St Kilda, Scotland (Nadler et al., 2000). The Soay sheep which are present on St Kilda are one of the most primitive sheep breeds currently in Europe and the species was believed to be widespread in the past, potentially throughout the 'Palearctic range' which included Europe, Russia, Asia and parts of North Africa and the Northern Arab peninsula. Previous researchers have hypothesised that Soay sheep may have been introduced to the St Kilda islands by the Norse and later, introduced to the Scottish border regions in the late 1800s providing a potential hypothesis for the introduction of *N. battus* to Britain however, no evidence of this could be observed in available DNA sequence phylogeny (Nadler et al., 2000). The observation of *N. battus* in Scotland a decade before it was recorded in Norway and evidence of the prior movement of sheep from Scotland to Norway also contradict this hypothesis. Given the lack of evidence regarding the movement of *N. battus* from the arctic to Britain, this is open for

speculation. It has been suggested that *N. battus* may have originated from deer due to morphological similarities between *N. battus* and *N. roscidus* (Jansen, 1973). Phylogeographic analysis of deer populations on the outer and inner Hebridean islands of Scotland, mainland UK and nearby Scandinavian countries suggests that these populations are unlikely to be ancestrally linked, leading the authors to hypothesise that deer and other livestock were historically transported long distances by boat (Stanton et al., 2016). The introduction of *N. battus* to the UK may therefore not have been direct and may remain a mystery.

The presence of *N. battus* in isolated foci in Italy and Yugoslavia may have resulted from recent animal introductions from the UK or could suggest that the distribution of *N. battus* was much wider in the past. *N. battus* may potentially have been present across the Palearctic range and since become isolated and expanded (Nadler et al., 2000), perhaps linked with the suggested historic distribution of red deer (Meiri et al., 2013). *N. battus* was reported in Southwest Norway in 1961 following the introduction of Suffolk sheep from the Scotland several years before (Helle, 1969). It is likely that only a small number of parasites were introduced, resulting in a slow build-up of *N. battus* with common mountain grazing systems spreading infection between flocks. Following the introduction of *N. battus* to Norway, it took over from *N. filicollis* and *N. spathiger* as the dominant species (Helle, 1969), the success of this species over the others was thought to be due to the differences in life history traits, particularly *N. spathiger* which is active throughout the year (Boag and Thomas, 1975). By 1989, *N. battus* had been documented in Italy, France, the Netherlands, USA, Canada and Yugoslavia (Borgsteede, 1983; Borgsteede and Konig, 1979; Hoberg et al., 1986; Hubert and Kerboeuf, 1985; Rickard et al., 1989; Smith and Hines, 1987). Introductions of this species to Europe and Canada and secondary translocations to several regions of North America can be traced to the introduction of adult sheep from the UK which may have been harbouring low level *N. battus* burdens (Rickard et al., 1989). A study carried out in 2000

found that isolates from Canada and North America showed minimal genotypic variation and were similar to those found in the UK indicating a relatively recent introduction and a potential founder effect following translocation (Nadler et al., 2000). Interestingly, despite the genetic similarity between the isolates from North America and Britain, the epidemiology of the isolates was significantly different. North American isolates hatched in late autumn and early winter rather than spring, typically causing low level infection in older animals (Rickard et al., 1989). Differential behaviour of genotypically similar isolates at different locations suggest that environmental conditions may be driving the epidemiology of this species, as previously suggested (Thomas, 1990).

1.5 Epidemiology

Early characterisation studies of UK isolates described the restricted seasonality of *N. battus*, detailing acute spring infection following mass-hatching events between April and June. Despite the high pasture contamination in spring, the number of larvae on pasture dropped quickly and *N. battus* was observed to transmit from one year's lamb crop to the next via eggs which remained intact on pasture throughout the year for hatching the following spring (Boag and Thomas, 1975; Thomas, 1959b; Thomas and Stevens, 1956).

Environmental factors are believed to govern the hatching of *N. battus* eggs, particularly temperature (as discussed in section 1.1), it remains unclear whether additional factors are also involved. Field studies carried out in the late 1980's highlighted significant variation in the magnitude and timing of egg hatching between isolates and under different environmental conditions (Thomas, 1990), further supporting the hypothesis that hatching is controlled by temperature and other climatic factors. However, variation in the timing and magnitude of egg hatching in different *N. battus* isolates placed under the same environmental conditions was also observed. The variable hatching may indicate local adaptation of *N. battus* isolates to specific climatic conditions or, that environmental

conditions are not the sole factor involved. Thomas (1990) highlighted the complex nature of egg hatching given the 'typical' *N. battus* epidemiology however, in recent years infection has become even more varied with increasing reports of clinical symptoms throughout summer and autumn (Sargison et al., 2012).

Figure 1.5 shows the occurrence of *N. battus* throughout 2015/16 in faecal samples submitted to the Animal and Plant Health Agency (APHA) parasite survailence centres for diagnostic analysis and highlights the detection of *N. battus* eggs in samples submitted in every month of the year. The greatest parasite challenge was found in spring, as historically documented (Boag and Thomas, 1975; Gibson, 1963; Thomas, 1959b). The observation of eggs in faecal samples throughout the year, a secondary peak of infection in autumn and clinical cases of *Nematodirosis* in older animals (Sargison et al., 2012) appears to represent a novel change in the epidemiology of this species. This pattern may change year on year. The novel disease timings observed on some UK farms resembled the epidemiology of American isolates of *N. battus* where peak hatching occurs in late autumn/early winter rather than spring (Rickard et al., 1989). As a result of the altered timing of infection, clinical cases of *nematodirosis* as a result of *N. battus* are rarely observed in North America as the peak of transmission occurs before lambing.

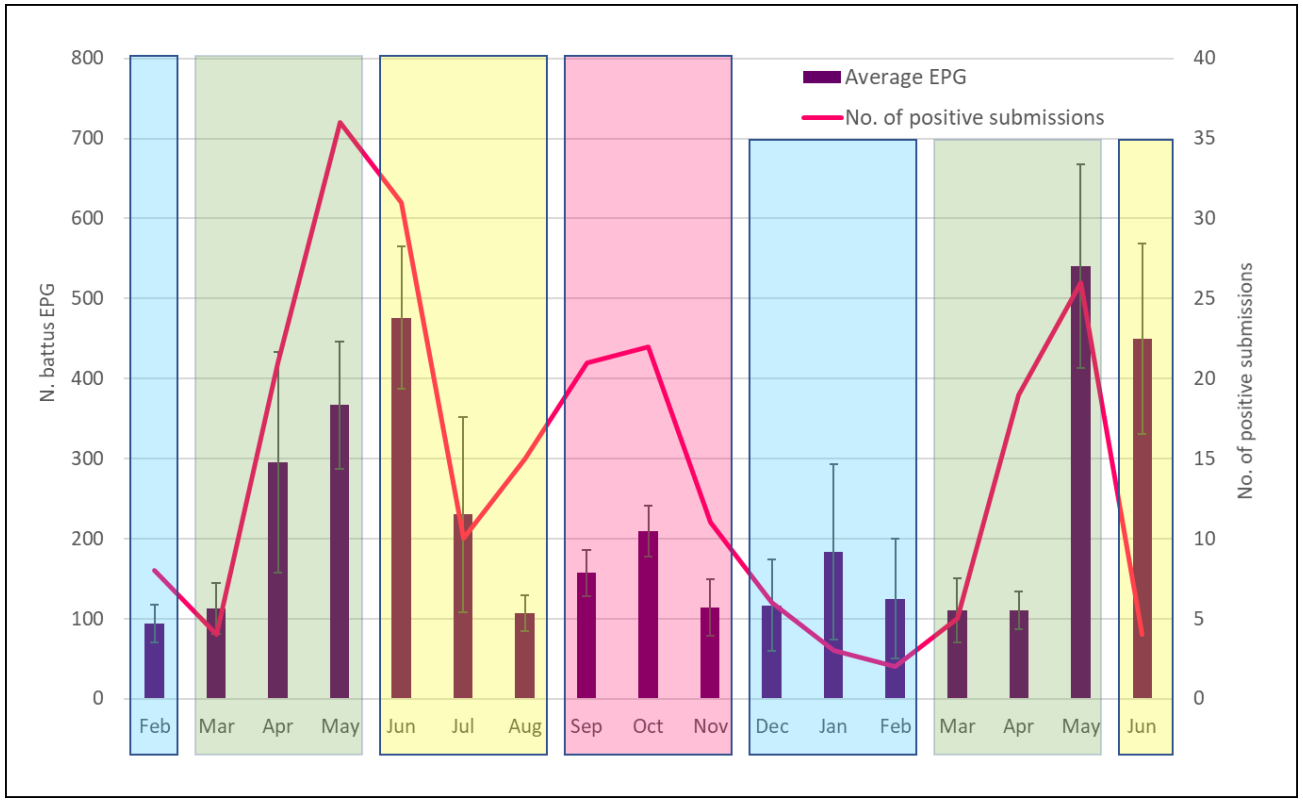


Figure 1.5. Parasite surveillance data from the Animal and Plant Health Agency.

Data showing the incidence of N. battus in diagnostic samples submitted throughout 2015/16. The bars represent the mean N. battus faecal egg count each month with error bars indicating the standard error of the mean (SEM) and the red line represents the number of submissions positive for N. battus each month. The blue, green, yellow and red boxes highlight the seasons (winter, spring, summer and autumn respectively).

A small proportion of eggs hatching in autumn was recorded in early characterisation studies (Boag and Thomas, 1975; Gibson, 1963; Thomas, 1959b) however, focus was placed on the spring populations. Several hypotheses have been offered in the literature of possible drivers for the increased autumn hatching, largely focused around climate change. Variation in spring temperature out-with the optimal range was found to interrupt egg hatching, which resumed once the conditions became optimal again (van Dijk and Morgan, 2008), suggesting that rapid temperature changes may not allow eggs to complete hatching within the typical spring window (van Dijk et al., 2008). Increased variability in temperature or shifting weather patterns as a result of climate change could shift the timing of egg hatch therefore introducing a possible mismatch between lamb grazing and larval availability on pasture (Gethings et al., 2015). Variation may select for hatching at a different time of year to safeguard transmission of the population (Van Dijk and Morgan, 2010). Previous studies have also highlighted the ability of some *N. battus* eggs to hatch successfully without a chill stimulus (Ash and Atkinson, 1983; van Dijk and Morgan, 2010). Therefore a proportion of eggs may not be restricted to over-wintering on pasture and thus, could hatch within the same year as they were deposited, i.e. hatching in autumn upon completion of development during the summer months. The mechanism of eggs hatching without prior chilling and the prevalence of this phenomenon has not previously been studied in detail but appears to be subject to variation at the isolate level (van Dijk and Morgan, 2010).

The aparent shift in epidemiology may benefit the parasite, for example, by circumventing control strategies implimented by farmers in spring. Hatching of eggs throughout the year would also likely result in a lower challenge at any given timepoint. Low level infection would not trigger the development of strong immunity or the characteristic hypersensitivity reaction typically observed in spring, therefore leading to more chronic infection and ultimaltely allowing adult female worms to pass out eggs over a longer period of time.

1.6 Predictive models of *N. battus* risk

As described above, parasite epidemiology is becoming increasingly variable, potentially in response to ongoing climate change, subsequent changes in farm management or further intensification within the livestock sector. In order to design and test sustainable control measures, it is important to understand and predict parasite behaviour. Transmission models are built using empirical data of the parasite lifecycle, infection dynamics and host:parasite interactions extracted from experimental and field trials. Transmission models have been developed for a wide range of human, veterinary and plant pathogens (Jeger et al., 1998; Kinsley et al., 2016; Wallace et al., 2014) and are beginning to be applied to GIN infections in ruminants (Rose et al., 2015). The models have been used for predicting the impact of climate change on parasite epidemiology, the identification of high risk exposure periods (Gethings et al., 2015; Vineer et al., 2016) and providing simulation models for testing novel control strategies (Rose et al., 2015).

The first widely available prediction model for livestock parasites in the UK identified high risk periods based on the link between soil temperature and egg hatching. This monthly parasite forecast remains in use currently (NADIS, 2018), allowing farmers to alter management strategies during high risk periods. In recent years, the availability of detailed information on farms has increased with the advancement of high-throughput diagnostics, increased monitoring and the introduction of mandatory electronic identification tags (EID) for sheep in the UK, amongst other factors. Greater access to information has driven the development of more detailed and precise models, increasing the power and reliability of predictions and simulations produced (Verschave et al., 2016). One such prediction model has been developed for *N. battus* and is available online as a free resource for farmers to monitor the risk of *N. battus* egg hatching in real-time (Stubbings, 2018). The model has been built using detailed information on egg development time and optimal climatic ranges for

development, hatching and larval survival (van Dijk et al., 2009; van Dijk and Morgan, 2008). Combining detailed parasite knowledge with real-time climatic data from weather stations and forecasts to predict when *N. battus* eggs are likely to hatch on pasture. Models such as the *N. battus* prediction model could assist on farm decision making, optimising treatment timings and management to minimise production losses due to parasitic infection.

1.6.1 Risk factor analysis

Further use of modelling in the study of biological systems is to explore correlations and risk factors within complex systems. For example, in farming systems there are a multitude of factors which vary both within and between farms, it is therefore difficult to compare cases in order to determine risk factors associated with a specific outcome. Generalised linear mixed effect models (GLMMs) are often employed to study risk factors in biological systems as these models can account for random and fixed effects and over-dispersion observed in binary data. Random effects are factors which are a source of random variation but which are not specifically of interest e.g. individuals from a large population. Fixed effects could be described as factors of interest or variables which could be manipulated or repeated during experiments. Mixed models take both fixed and random effects into account and describe the amount of variation in the outcome variable attributed to each fixed effect. This modelling approach has been used to investigate risk factors associated with a number of human and veterinary diseases (Yatabe et al., 2011; Yitshak-Sade et al., 2017). A GLMM approach was used to explore risk factors associated with GIN infection in small ruminants in Kenya; the model included parasite, climatic and management factors, highlighting grazing strategy, recent anthelmintic usage and farmer education level as important factors determining the magnitude of faecal egg counts (FEC) in sheep and goats (Odoi et al., 2007). The information fed into these models is typically collected by a combination of sensors, for environmental factors, and questionnaire surveys on management factors and human behaviour. Management and attitudinal questionnaires have been widely used to gather

detailed information on farm practices and to study what influences decision making by farmers (Burgess et al., 2012; Jack et al., 2017; Morgan et al., 2012). As management can have a significant impact on parasite population dynamics, it is important to understand the practices currently in use to inform the development of applicable control strategies and understanding the drivers of behaviour is key to enhancing future uptake of recommendations. A recent study identified that the key drivers of uptake of novel practices by farmers were age, education level and perception of the risk of AR threatening their sheep production enterprise (Jack et al., 2017) therefore, advice and recommendations can be targeted to the cohort of farmers most likely to respond.

1.7 Control strategies

1.7.1 Management

Farmers have long since been advised to control *N. battus* by alternative grazing methods. *N. battus* is generally transmitted from one year's lamb crop to the next by repeated grazing of young lambs on the same fields each spring (Boag and Thomas, 1975). Grazing strategies aimed at breaking the parasite life cycle, either by avoiding grazing infected fields every second year or alternate grazing of lambs and cattle each year have been shown to reduce contamination significantly (Black, 1964; Boag and Thomas, 1975). There are several reports of *N. battus* eggs remaining on pasture for 2 years before hatching which have been shown to produce viable infections in lambs (Boag and Thomas, 1975; Gibson, 1963) however, despite the persistence of a small proportion of eggs the reduction in parasite burden was found to be significant (Boag and Thomas, 1975). Adult cattle are typically immune to infection with *N. battus* however, rotational grazing of lambs and young claves was found not to interrupt the transmission of this parasite as calves can develop clinical *nematodirosis* given a large challenge and shed high numbers of eggs onto pasture (Bairden and Armour, 1987).

With the rapid development and spread of anthelmintic resistance in GIN species other than *N. battus*, alternative control measures such as grazing and pasture management are gaining importance to minimise dependence on anthelmintics. Grazing management systems are based around the parasite lifecycle and grass growth to avoid grazing contaminated paddocks when eggs are likely to hatch and to maximise the potential energy from grass growth. Rotational grazing, where animals are grazed on small paddocks for a number of days before being moved to fresh paddocks, has been shown to be effective in tropical climates where larvae only remain viable on pasture for a short period of time (Barger et al., 1994) by reducing the larval challenge to grazing ruminants. In temperate regions where GIN larvae have been shown to remain viable on pasture for up to two years (Baker, 1939), rotational grazing has been shown to have a lesser impact (Jackson and Miller, 2006).

Reseeding pasture increases the yield and quality of grazing whilst reducing parasite contamination. New ley pasture has been shown to produce up to 5 tonnes more feed per hectare than long-term permanent pasture (Teagasc, 2014). Although a small proportion of GIN can persist following reseeding, the contamination level is significantly reduced. Forage type has also been shown to affect parasitism with increased white clover content having a positive impact on lamb performance throughout the grazing season (Niezen et al., 2002a). Feed and forage has also been demonstrated to influence the development and survival of nematode larvae in faeces (Marley et al., 2003) and faecal moisture content which is an important factor of faecal breakdown and subsequent egg development rates (Marley et al., 2003; Niezen et al., 2002b).

1.7.2 Nutrition

Pasture management is important in maximising the nutrition of grazing animals as varied grazing strategies have been shown to influence pasture composition (Morley et al., 1969). For example, rotational systems have been shown to favour perennial species such as white clover which have a higher crude protein content than ryegrass (Morley et al., 1969; Rattray and Joyce, 1974).

Links between nutrition and immunity/resistance to GIN infection have also been demonstrated, both in livestock and humans (Coop and Kyriazakis, 1999, 2001). Nutrient allocation in sheep is believed to favour system maintenance, growth and reproduction over immune expression given restricted protein availability (Coop and Kyriazakis, 1999). The impact of crude protein supplementation on GIN infection and the development of immunity is complex. Coop and Kyriazakis (1999) developed a framework to describe the allocation of nutrient resources, concluding that protein intake had little impact on the early rate of acquisition of immunity to parasites in young lambs. Nematode establishment rates and time taken for the immune response to significantly impact parasitic burden were found to be independent of host nutrition in multiple studies (Dobson and Bawden, 1974; Kyriazakis et al., 1994; van Houtert et al., 1995). However, substantial evidence exists for improved expression of immunity in sheep, particularly lambs, given protein supplementation, suggesting that cellular responses and antibody levels are increased in animals on high protein diets (Dobson and Bawden, 1974; Kambara et al., 1993; Kyriazakis et al., 1994; van Houtert et al., 1995). Studies have illustrated significant increase in adult worm expulsion and reduced fecundity in GIN leading to significant reduction in faecal egg count given increased nutrition (Kambara et al., 1993; van Houtert et al., 1995; Wallace et al., 1995). Growth in lambs appears to be prioritised over immune expression therefore immune response will be greatly influenced by the nutritional level in the host (Coop and Kyriazakis, 2001).

1.7.3 Chemical

Alternative grazing and pasture management strategies for nematode control cannot be implemented on all farms due to farm layout, topography and differing farm enterprises therefore chemical control is often relied upon. There are five classes of anthelmintic compounds licensed for use in the UK (Table 1.1) with many formulations of each on the market.

Table 1.1. Anthelmintic compounds currently licensed for use in the UK, the year each was introduced to the market and the parasites targeted by each class.

Active compound	Code	Colour	Year of introduction	Active against
Benzimidazole	1-BZ	white	1961	Roundworms, lungworm and tapeworm (some formulations also effective against adult liver fluke)
Levamisole	2-LV	yellow	1970	Roundworms and lungworm
Macrocyclic lactones	3-ML	clear	1981	Roundworms and lungworm (some formulations also effective against sheep scab)
Monepantel	4-AD	orange	2009	Roundworms
Derquantel & Abamectin	5-SI	purple	2009	Roundworms and lungworm

Due to the variability in the timing of egg hatching in *N. battus*, particularly in recent years, and the lack of prolonged activity of 'long-acting' products against this species, it is difficult to time the use of anthelmintic treatment to prevent clinical signs in lambs. Treatments administered to control this species are therefore typically aimed at treating disease and minimising egg output onto pasture, reducing the challenge for the following year's lamb crop.

1.7.3.1 Benzimidazoles (1-BZ)

This thesis will focus on Benzimidazole (BZ) as these compounds are typically administered to control *N. battus* using blanket treatment of lambs in spring. BZ compounds are generally favoured over other anthelmintic classes for the control of this parasite species due to their high safety index; making them relatively ideal for treatment of young stock (Lacey and Gill, 1994). BZ has also exhibited a continued high efficacy against *N. battus* until the recent emergence of resistance in this species (Mitchell et al., 2011). Many BZ formulations have been launched for the control of GIN in ruminants and are effective against both adult and immature stages of *N. battus* and other GIN species, certain formulations are also effective against adult *Fasciola hepatica* when administered at a higher dose.

1.7.3.1.1 Mode of action

BZ compounds interact with the colchicine-binding domain of tubulin (Figure 1.6), inhibiting the dynamic polymerisation and depolymerisation of microtubules (Lacey, 1988, 1990). The target tubulin protein is highly conserved across taxa however, BZ compounds were found to possess a high affinity specifically for parasite tubulin at 37^{o}C providing the characteristic low host toxicity observed with these compounds (Lacey and Gill, 1994). Several intrinsic cellular processes rely on the formation of microtubules including cellular division and the migration of sub-cellular organelles (McKellar and Scott, 1990). Benzimidazole compounds have also been associated with interruption of the metabolic pathways of nematodes through the inhibition of the fumarate reductase system and glucose transport (Barragry, 1984; Prichard, 1973). Phenol and salicylanilide groups in the benzimidazole structure inhibit oxidative phosphorylation within the mitochondrial electron transport system which is coupled with the reduction of fumerate to succinate; a fundamental process in nematode anaerobic metabolism (Bryant and Bennet, 1983; Prichard, 1973). However, a reduction in

fumerate reductase activity was not observed in all BZ-resistant strains of *H. contortus* (Bryant and Bennet, 1983), suggesting that this is not the central mechanism of action of benzimidazole and that perhaps multiple processes occur simultaneously. Overall, BZ compounds interfere with energy production within parasites, leading to death by starvation.

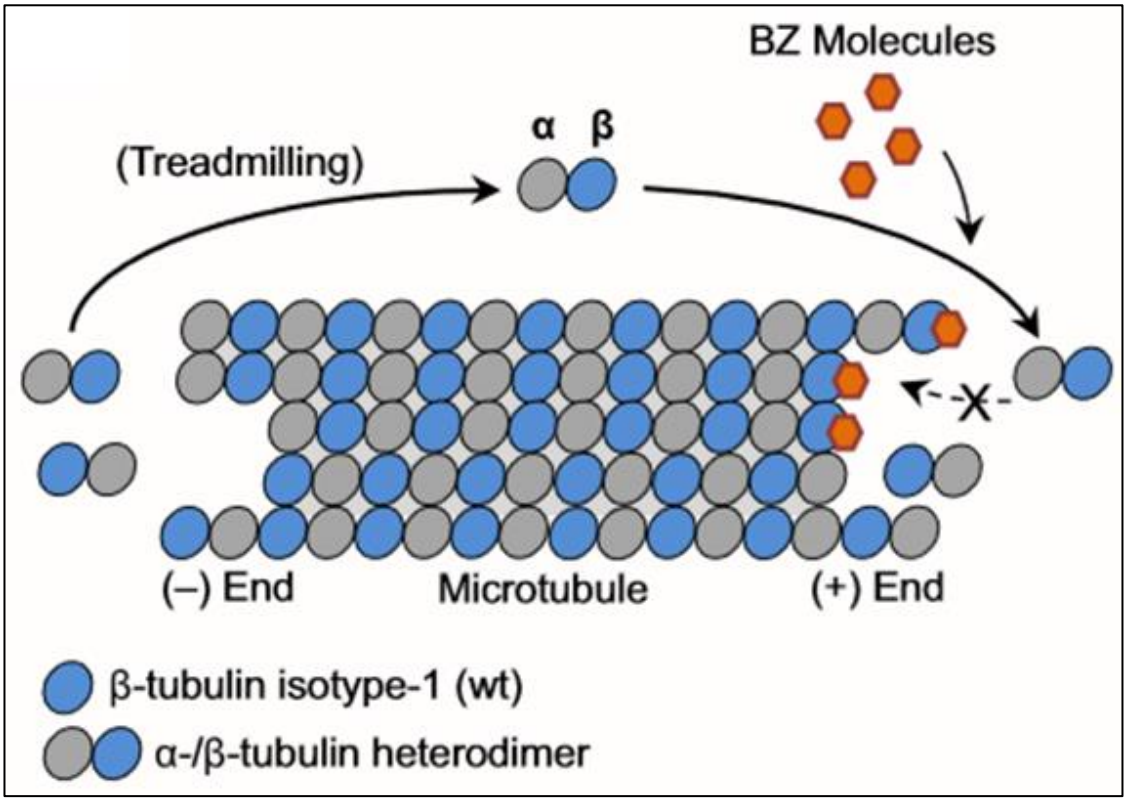


Figure 1.6. Diagram of the mode of action of benzimidazole.

Diagram shows the interaction of benzimidazole compounds with colchicine binding domains on tubulin molecules preventing polymerisation and depolymerisation of microtubules. Figure adapted from (Whittaker et al., 2017) with permission from John Wiley and Sons publishers.

1.7.3.2 Mechanisms of resistance

The genetic basis of BZ-resistance has been extensively studied in several GIN species and was found to be linked to a series of single nucleotide polymorphisms (SNPs) at sites on the β-tubulin isotype 1 gene (Ghisi et al., 2007; Kwa et al., 1994; Silvestre and Cabaret, 2002) and a reduction in genetic diversity through the selective loss of β-tubulin isotype 2 genes (Kwa et al., 1993). SNPs at codons 167, 198 and 200 have been shown to confer resistance by altering the confirmation of the colchicine-binding site of parasite tubulin, thus lowering the binding affinity of BZ drugs to parasite tubulin (Lacey, 1988). As discussed above, the mechanism of action of benzimidazole compounds is not fully understood therefore additional mechanisms may also play a role in the action of these drugs and resistance to them. The prevalence of the β-tubulin SNPs appear to be the major mechanism and well correlated with phenotypic resistance (von Samson-Himmelstjerna et al., 2002).

The SNP at codon 200 (F200Y) codes for an amino acid change from phenylalanine to tyrosine (TAC-TTC) (Kwa et al., 1994) and is believed to be the principal mechanism of phenotypic BZ-resistance in the majority of ovine nematode species studied to date (Kwa et al., 1994; Ramunke et al., 2016). F200Y has also been reported in human and plant pathogens (Jung et al., 1992; Koenraadt et al., 1992). The mutations at codons 167 and 198 code for amino acid changes from phenylalanine to tyrosine (TAC-TTC) and glutamic acid to alanine (GAA-GCA) respectively (Ghisi et al., 2007; Silvestre and Cabaret, 2002). The three SNPs are often found in combination i.e. one individual parasite can carry mutations at position 200 and 167. However, the SNPs appear to be mutually exclusive in all nematode species studied to date. Double heterozygous individuals have been identified but double homozygous resistant individuals appear generally to be a lethal combination. All three polymorphisms have also been identified in fungal species where they confer resistance to BZ-related benomyl compounds (Jung et al., 1992; Koenraadt et al., 1992; Lila et al., 2003). The prevalence and frequency of the SNPs vary between nematode species and country of origin. F167Y and

E198A have been identified at high frequency (Ramunke et al., 2016; Redman et al., 2015) but are typically less common in sheep nematodes, particularly in European isolates, compared to the F200Y mutation. Isolates from out-with Europe have typically been found to have higher F167Y and E198A allele frequencies (Chaudhry et al., 2009; Chaudhry et al., 2015b). Despite the high prevalence of F200Y, studies have suggested that F167Y and E198A may confer a more resistant phenotype compared to F200Y (Kotze et al., 2012). Patterns have been observed in the development of resistance in *Teladorsagia circumcincta*, suggesting that F200Y may predominate primarily but as the population becomes more phenotypically resistant, the frequency of F200Y decreases with increasing F167Y (Kotze et al., 2012) though it is yet to be determined whether this is a commonly observed phenomena.

1.7.4 Benzimidazole-resistant *N. battus*

N. battus was previously believed to be refractory to the development of BZ-resistance due to the continued high efficacy of these compounds against this species despite repeated use to control infection in lambs each spring for over 50 years. The first case of BZ-resistance in *N. battus* was detected in 2010 on a commercial farm in Northern England when faecal samples were submitted samples to the APHA parasite surveillance centre for analysis following treatment failures (Mitchell et al., 2011). The *N. battus* population was isolated and used to experimentally infect lambs at Moredun Research Institute to conduct a controlled efficacy test (CET). Lambs were infected with either the suspect-resistant isolate or a BZ-susceptible lab isolate, a proportion of each group were treated with fenbendazole and the faecal egg counts and adult worm burdens were compared between treated and untreated lambs *post mortem*. The BZ-resistance case was confirmed and material was collected for molecular characterisation of the resistant isolate (Morrison et al., 2014). Preliminary characterisation of the initial BZ-resistant *N. battus* isolate identified the F200Y SNP at around 80% resistant allele frequency in worms which had survived fenbendazole treatment

(Morrison et al., 2014). The results of preliminary investigations by Morrison *et al*. (2014), suggest that F200Y likely conferred BZ-resistance in *N. battus*. The survival of a small number of worms which were homozygous susceptible at codon 200 (TAC/TAC) suggested that an alternative mechanism may also be used by this species. Subsequent unpublished work suggested that survivors carried the resistance genes at codon 167 instead (Morrison 2018, *personal communication*).

1.7.5 Origins of benzimidazole resistance

Several hypotheses exist regarding the potential mechanism of the emergence and dissemination of SNPs associated with BZ-resistance in nematodes (Gilleard and Beech, 2007). Firstly, the mutations may have arisen once, expanded due to selection pressure and disseminated from a single source. Secondly, the mutations may have pre-existed within the parasite population and expanded as selection pressure was introduced or, thirdly, the same mutation could have repeatedly arisen in multiple locations and disseminated from each locus. The method of the emergence of resistance can be studied from the genetic structure of the population. Expansion of the resistant allele from a single source would provide a hard selective sweep with restricted diversity as the mutation would have occurred on a single haplotype. With multiple origins, haplotype diversity would be high with each resistant haplotype being more closely linked phylogenetically to a susceptible haplotype than to other resistant haplotypes. The recurrent mutation and pre-adaptive theories would both result in 'soft selective sweeps' in which each farm population would likely include multiple resistant haplotypes (Redman et al., 2015).

Many studies have been conducted to investigate the potential origins of BZ-resistance in trichostrongylid species. Genetic evidence of both hard and soft selective sweeps have been demonstrated, dependent upon the SNP, parasite species and country studied. Multiple independent studies of *H. contortus* and *T. circumcincta* have concluded that F200Y is likely

to have arisen through recurrent mutations in different locations. Phylogenetic evaluation of BZ-resistant *H. contortus* in southern India provided evidence for at least three independent origins of F200Y in 28 populations (Chaudhry et al., 2015b). A UK study of *T. circumcincta* identified five resistant haplotypes on seven farms, suggesting at least four independent origins of the SNP (Redman et al., 2015). Comparison of the resistant haplotype diversity observed in *H. contortus* and *T. circumcincta* identified differences in haplotype allocation between populations despite a high overall haplotype diversity in both species, i.e. hard and soft selective sweeps, likely due to disparities in the life history traits of these two species (Redman et al., 2015). *H. contortus* is a tropical species and is less well adapted to the challenging conditions of UK winter and so, restriction of the population during winter months is likely to result in population bottlenecks, leading to re-expansion of the population from a limited number of surviving haplotypes (Redman et al., 2015; Silvestre et al., 2009). Unlike F200Y, which has been invariably present in studies worldwide (Barrere et al., 2013; Brasil et al., 2012; Chaudhry et al., 2015a; Ramunke et al., 2016), mutations at positions 167 and 198 appear to be restricted by parasite species and country. The restricted prevalence and results of phylogenetic analysis of F167Y and E198A suggest that these are rare mutations disseminated from a single source (Chaudhry et al., 2015a; Redman et al., 2015).

1.7.6 Drivers of benzimidazole resistance

As well as life history traits of the parasite species, farm management practices can have a significant impact on GIN population dynamics, influencing the evolution of parasite behaviour and the development of anthelmintic resistance. Table 1.2 provides a summary of factors which have been highlighted in the literature as potential risk factors for the development of AR in trichostrongylid nematodes although this is not an exhaustive list.

The frequency and timing of anthelmintic treatment have been highlighted by several authors as key risk factors in the development of AR in trichostrongylid species (Falzon et al.,

2014; Suarez and Cristel, 2014; Suter et al., 2004; Vadlejch et al., 2014), placing direct selection pressure on parasite populations by screening for resistant alleles within the population. Other than the frequency of anthelmintic treatment, there appear to be two other key drivers of anthelmintic resistance in GIN; under-dosing (Calvete et al., 2012; McMahon et al., 2017) and the maintenance of a refugia of anthelmintic susceptible parasites (van Wyk, 2001).

Under-dosing animals can allow heterozygous resistant parasites to survive treatment, increasing the resistant allele frequency within the population (Roush and McKenzie, 1987). A recent study of Northern Irish farmers identified that the majority of farms surveyed (51%) were at risk of over- or under-dosing due to animal weight estimation and 69% of responders did not routinely calibrate dosing equipment (McMahon et al., 2017). Incorrect dosing technique can also lead to under-dosing via closure of the oesophageal groove; a swallowing reflex which results in the drug dose by-passing the rumen and entering directly into the abomasum, preventing the correct metabolism of the drug into active compounds and reduced uptake (Prichard and Hennessy, 1981). Diet and co-current infection with diarrhoea-causing agents have also been highlighted as potential mechanisms of under-dosing as increased flow of digesta through the gut can lower absorption of the drug (Ali and Hennessy, 1993). The use of long-acting anthelmintic compounds is common in ewes around lambing to reduce pasture contamination during the peri-parturient relaxation in immunity however, these treatments have been associated with under-dosing, both in ewes and lambs as the drug concentration in ewes reduces over time and lambs can be exposed to small amount of drug transfer in milk (Leathwick et al., 2015).

Parasite refugia describes the proportion of the nematode population which is unexposed to anthelmintic selection pressure and which is available to re-infect treated animals, i.e. parasites on pasture and the population which resides within any untreated animals or

wildlife reservoirs (van Wyk, 2001). The maintenance of a diverse refugia has been shown to slow the progression of AR development in trichostrongylid species (Kenyon et al., 2013). Resistant individuals surviving treatment are diluted by reinfection of the hosts with susceptible nematodes from pasture, reducing the mating potential and competitive advantage of resistant parasites and reducing the proportion of resistant eggs contaminating pasture. 'Dose and move' practices where animals are moved onto low contamination pasture immediately after treatment have been highlighted as increasing the odds of AR development and illustrates the theory well (Falzon et al., 2014; Vadlejch et al., 2014). Treated animals are not re-infected with susceptible nematodes due to the low number of L_3 present on the 'clean' pasture, therefore eggs produced by the resistant nematodes which survived treatment within the animals will be passed out and form the basis of the pasture population (Falzon et al., 2014; Hughes et al., 2007; Suter et al., 2004). Parasite control strategies have been developed to maximise refugia of trichostrongylid species throughout the grazing season such as targeted selective treatment where a proportion of resilient animals are left untreated to maximise the nematode population un-exposed to anthelmintic treatment (Kenyon et al., 2013; Melville et al., 2016). GIN are seasonal parasites and as such, refugia also varies seasonally. Anthelmintic treatments administered at times when parasite refugia is naturally low, such as autumn/winter provide a significantly greater impact on AR development than treatments administered during summer due to the lower rate of re-infection (Suter et al., 2004). Adult sheep are often treated in autumn, around mating (Morgan et al., 2012), and at lambing to reduce the egg output by ewes during the peri-parturient relaxation in immunity (Leathwick et al., 2006), both times of naturally low parasite refugia therefore, these treatments have been suggested as potential drivers of resistance. Not only are anthelmintic treatments at mating and lambing administered at periods of low parasite refugia, but also the establishment rate of incoming larvae is

considerably lower in adult sheep compared to lambs therefore, re-infection and dilution of resistant parasites is lower (Leathwick et al., 2006; Leathwick et al., 1999).

Parasite refugia has been well defined for trichostrongylid species however, the presence and impact of this in *N. battus* is unclear due to the significant differences in life history traits between the species. Given the typical *N. battus* lifecycle, synchronous egg hatching in spring and fast larval decay on pasture means that the window for re-infection of treated animals is short and the majority of the population which will be transmitted to the following year exists within the animals. *N. battus* 'refugia' may therefore consist only of the small population within untreated animals, namely adult sheep, eggs passed out by lambs prior to treatment and potentially unhatched eggs on pasture, dependent upon when those eggs are predisposed to hatch. The influence of this small un-exposed population on the development and dissemination of anthelmintic resistance remains unknown and may form the basis of future studies.

Grazing strategy can have a significant influence on parasite population dynamics by varying the availability of suitable hosts for infection and pasture contamination rates throughout the grazing season. Despite animal movements having no direct selection pressure for anthelmintic resistance, grazing strategies have been highlighted as potential drivers in trichostrongylid species due to their impact on transmission (Leathwick et al., 2009; Odoi et al., 2007). Similar to parasite refugia, the influence of grazing strategy on *N. battus* population dynamics and the development of anthelmintic resistance in this species requires investigation.

Alternatively, resistant parasites could be introduced onto the farm from new or returning stock (Coles and Roush, 1992). Given the high prevalence of AR in UK nematode populations and significant animal movement throughout the country and beyond, effective quarantining of new and returning stock has been identified as a protective factor in the development of

resistance (Lawrence et al., 2006). The Sustainable Control of Parasites in Sheep (SCOPS) recommend that animals should be treated with at least one of the new anthelmintic classes (4-AD or 5-SI) and animals should be kept separate from existing stock for a minimum of 21 days (Abbott et al., 2012). Some GIN species, including *N. battus*, can also infect wildlife hosts, particularly deer and rabbits (Boag, 1972; Dunn, 1965) and perhaps migratory waterfowl (Hollands, 2018) which are regularly observed grazing livestock fields. Deer have been shown to harbour anthelmintic resistant GIN, capable of infecting sheep and cattle (Chintoan-Uta et al., 2014) therefore, providing another possible route of transmission and dissemination of AR for *N. battus* and other GIN species.

Table 1.2. Farm management, parasite and host factors previously associated with the development of anthelmintic resistance in sheep nematodes.

Farm management	**Parasite**	**Host**
Quarantine/introduction of resistant alleles onto farm via animal movement	low refugia	Pharmacokinetics of the anthelmintic
Grazing strategies	Biotic potential	Digesta flow-rate/feeding
Maintenance of refugia	Resistant allele frequency at onset of treatment	
Under dosing/dosing technique	Minimum generation time	
Inappropriate timing of treatment	Adult parasite and larval longevity	
Frequency of treatment		
Inappropriate storage/use of out-of-date anthelmintics		

1.7.7 Detection of anthelmintic resistance

1.7.7.1 In vivo

The 'gold standard' test for anthelmintic resistance is the controlled efficacy 'dose and slaughter' test where animals are infected with the parasite(s) of interest, anthelmintic treatment is administered following establishment. The animals are then slaughtered 7-14 days post treatment and the parasitic worm burden is assessed *post mortem*. Although this method gives a definitive measure of anthelmintic efficacy, it is not an appropriate method for regular anthelmintic efficacy testing on farm given the animal and labour costs. The faecal egg count reduction test (FECRT) is a more usable *in vivo* test. Animals are treated with the anthelmintic of interest and faecal egg counts are compared on the day of treatment and 3-14 days post-treatment dependent upon the anthelmintic used and the parasite species of interest (Coles et al., 1992). Anthelmintic resistance was characterised by the world association for the advancement of veterinary parasitology (WAAVP) as a reduction in faecal egg count by less than 95% with a lower confidence interval of less than 90% (Coles et al., 1992). FECRT is simple to perform however, in acute *N. battus* infection, worm burden can be reduced rapidly due to hypersensitivity reactions within the host. It can therefore be difficult to determine whether egg reduction was due to effective treatment or the host immune response. FECRT could therefore underestimate BZ-resistance in this species. The time between samples and repeated animal handling also make FECRT labour-intensive. Tests which could be conducted on a single sample without the need for anthelmintic treatment of animals would therefore be favourable.

1.7.7.2 In vitro

1.7.7.2.1 Biological assays

Several biological laboratory-based assays have been developed to test anthelmintic resistance in trichostrongylid nematode species, measuring the concentration of anthelmintic capable of inhibiting parasite activity. The parasite material (eggs or larvae depending on the test) is incubated in increasing concentrations of anthelmintic and the activity of the parasite is assessed (e.g. eggs hatched, larvae developed, migrated or fed). Each test is based on the mode of action of the drug; egg hatch assays (EHA) are typically used to test benzimidazoles and levamisole as these drugs interfere with the development of the larvae within the eggs (Coles and Simpkin, 1977; Le Jambre, 1976). Larval migration assays are suitable for testing ivermectin, benzimidazoles and levamisole as these compounds inhibit larval motility and development (Rothwell and Sangster, 1993).

Comparison of EHA and FECRT results conducted on the same populations have suggested that either method is suitable for the detection of BZ resistance however, FECRT was found to be a more sensitive test for trichostrongylid nematode species in sheep and horses (Craven et al., 1999; Grimshaw et al., 1994; Martin et al., 1989). Laboratory-based biological tests provide results within 3-5 days from a single sample and negate the need for anthelmintic treatment of animals or repeated gathering and sampling. These tests are not suitable for the study of BZ-resistance in *N. battus* though due to differences in the timing and triggers for egg hatching between this and other nematode species.

1.7.7.2.2 Molecular diagnostic tests

As the genetic markers of benzimidazole resistance are known, molecular tools can be used to detect and quantify the β-tubulin SNP mutations to give an estimation of BZ-efficacy within a population. Molecular diagnostics provide rapid, reliable information from a single sample without administering anthelmintic to a group of animals or re-gathering animals

post-treatment. Despite the lack of understanding of the correlation between resistant allele frequency and phenotypic resistance, the output gives an indication of how advanced resistance is within a population. There is currently no 'gold standard' method for the molecular detection and quantification of SNPs and many assays have been developed. The majority of SNP detection methods are two stage processes; amplification of the target region then detection of the mutation by recognition of fluorescence or the binding of sequence-specific probes e.g. pyrosequencing, deep amplicon sequencing and Luminex (Avramenko et al., 2018; Ha et al., 2007; von Samson-Himmelstjerna et al., 2009). Alternatively, sequence variation can be detected by selective amplification e.g. qPCR, allele-specific PCR or loop-mediated amplification (Fukuta et al., 2006; von Samson-Himmelstjerna et al., 2009). The above is not an exhaustive list of the platforms suitable for developing SNP detection tools and each has strengths and weaknesses which tailor their application. In the current project we focused on the development of assays using pyrosequencing, next generation sequencing and loop-mediated isothermal amplification (LAMP) for reliable SNP detection, the generation of extensive information and rapid, point-of-care results respectively.

1.7.7.2.2.1 Pyrosequencing

Pyrosequencing is widely used in both medical and veterinary research and diagnostics providing accurate and rapid sequencing over a small target range, ideal for SNP genotyping. Several pyrosequencing assays have been developed for the detection and quantification of SNPs in nematode species of veterinary importance including, BZ-resistance associated SNPs in *T. circumcincta, H. contortus, Trichostrongylus colubriformis and N. battus* (Morrison et al., 2014; Ramunke et al., 2016; Skuce et al., 2010; von Samson-Himmelstjerna et al., 2009). These SNP assays have been widely used to conduct genotype prevalence surveys worldwide (Esteban-Ballesteros et al., 2017; Ramunke et al., 2016).

The pyrosequencing method is summarised in Figure 1.7 (England and Pettersson, 2005). The target region surrounding the SNP of interest (~50bp) is amplified by PCR using one biotinylated primer and one conventional un-modified primer. Following amplification, the biotinylated amplicons are captured on streptavidin beads and denatured to allow the sequencing primer to anneal. A mix of enzymes are added to the reaction; ATP sulfurylase, luciferase, apyrase and DNA polymerase and a substrate mixture containing adenosine-5-phosphosulfate and luciferin. Amplification of the growing strand proceeds as in PCR, when each nucleotide is incorporated, inorganic phosphate is produced which is converted to ATP by the sulfurylase enzyme and adenosine-5-phosphosulfate. ATP is then converted to light by luciferase which is detected by the sequencer and signals the incorporation of a nucleotide, the intensity of the light signals the number of nucleotides incorporated. Only one type of nucleotide is released at a time and un-incorporated bases are removed by apyrase before the addition of the next nucleotide to the reaction therefore, the sequencer can record which nucleotide base has been added to the growing strand.

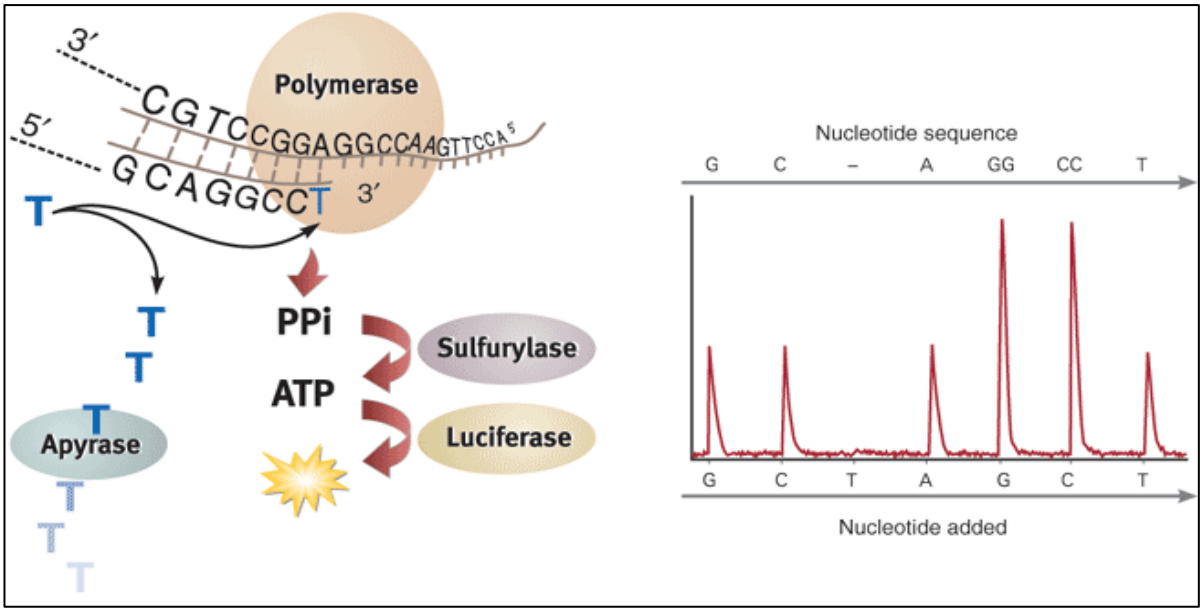


Figure 1.7. Overview of pyrosequencing methodology.

Figure adapted from (England and Pettersson, 2005) with permission from Springer Nature. The addition of each nucleotide during amplification of the template DNA sequence produces inorganic phosphate which is converted to ATP by the sulfurylase enzyme and adenosine-5-phosphosulfate. APT is then converted to light by luciferase which is detected and signals the incorporation of a nucleotide, the intensity of the light signal determines the height of the peak on the pyrogram and denotes the number of nucleotides incorporated. Unincorporated nucleotides are removed by apyrase before the addition of the next nucleotide to the reaction.

1.7.7.2.2.2 Next generation sequencing using Illumina MiSeq

Illumina MiSeq is a highly versatile deep amplicon sequencing technique, capable of whole genome and targeted sequencing such as diagnostics and SNP detection. Deep amplicon sequencing produces vast, detailed data sets, analysing up to 384 pooled samples within a single run using primers with unique barcoded sequences for data analysis. The bottleneck of deep amplicon sequencing is the development of suitable analysis pipelines to pull apart different populations, align and extract answers from the data. With recent technological advances, deep amplicon sequencing is becoming more accessible, making it suitable for both research and diagnostic testing. Assays and analysis pipelines have been developed for medical diagnostics such as typing of HPV viral strains (Nilyanimit et al., 2018) and diagnosis of respiratory infections (Thorburn et al., 2015). Assays have also been developed for veterinary use, most notably the development of the nemabiome assay which identifies nematode species from pooled eggs in faecal samples (Avramenko et al., 2015) and genotyping of BZ-resistance SNPs from a pool of different species (Avramenko et al., 2018). From a research perspective, in addition to the detection and quantification of SNP mutations, next-generation sequencing also provides a wealth of additional sequencing information which, given the development of appropriate analysis pipelines, could be mined for phylogenomic analysis (Yu et al., 2018) or screening for novel mutations (Chen et al., 2015).

Similar to pyrosequencing, MiSeq is also a sequence by synthesis method, covering a region around 450bp (Illumina, 2018), the method is outlined in Figure 1.8 (Illumina, 2018). The library preparation requires two PCR steps, i) to amplify the target region and ii) to attach sequencing adapters and barcodes unique to each sample population. Equal amounts of DNA from each sample are combined to produce a library which is then quantified, denatured and loaded onto the flowcell. The surface of the flowcell is coated in oligonucleotides, complimentary to the sequence adapters on the DNA amplicons. DNA clusters are created

when both ends of the amplicons bind the oligonucleotides on the surface of the flowcell, forming a 'bridge', amplification then occurs by sequential denature and amplification steps to form clonal clusters comprising thousands of copies of the original amplicon. For sequencing, fluorescently labelled nucleotides are added with the sequencing regents, as each base is incorporated into the growing strand, the wavelength and intensity of the light signal produced translates which nucleotide base was added.

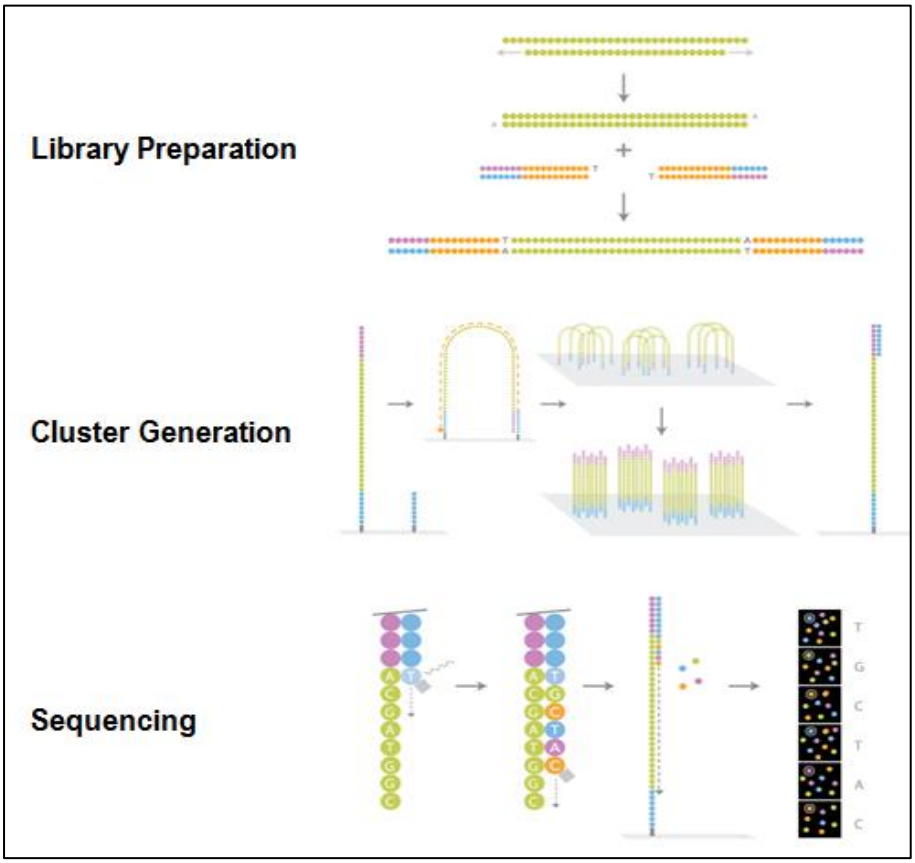


Figure 1.8. Illumina MiSeq next generation sequencing method overview.

Adapted from (Illumina, 2018). During library preparation, the sequence of interest (green) was amplified using primers (orange) with sequence barcodes specific to each population attached (pink/blue). Amplicons from each population were mixed in equal amounts and loaded onto the flowcell for cluster generation. Both ends of the DNA strands bind oligonucleotides on the surface of the flowcell (blue) to form 'bridges'. Clonal amplification of the bridge structures results in sequence clusters attached to the surface of the flowcell. Sequencing primers then bind and sequence by synthesis; as nucleotides are added to the growing strand, a fluorochrome signal is produced and recorded.

Each MiSeq run produces around 10,000 sequences per sample (Illumina, 2018) therefore analysis pipelines are required to manage the vast data sets and program the analysis. Pipelines contain a number of different programs which manage, manipulate and analyse the sequence data with computer code written to direct the data between the programs. Analysis typically begins with aligning the sequence files and 'cleaning' the data by removing nonsense reads. Sequences which pass the clean-up phase are then compared to a series of reference sequences, for example a reference library of nematode species and the reads can be grouped according to the reference sequences to which they align. Depending on the purpose of the analysis, SNP detection and quantification may then occur. Stringencies and inclusion criteria are set at each stage of analysis to control the integrity of the analysis.

1.7.7.2.2.3 Loop-mediated isothermal amplification (LAMP)

Lab-based technologies provide reliable, detailed results but, they require complex processing and transportation of samples to a diagnostic laboratory which inevitably delays the results. Significant advances have been made in the development of novel isothermal nucleic acid amplification technologies (iNAATs) in recent years. These techniques rapidly amplify small target regions of DNA and are often coupled with direct detection methods for example intercalating dyes, allowing for immediate detection of results without downstream processing (Oscorbin et al., 2016). These isothermal reactions have been developed as point-of-care devices for detection of a wide range of medical and veterinary infecting agents (Fang et al., 2011; Trinh and Lee, 2018; Waters et al., 2014). One of the most well studied and versatile iNAATs is loop-mediated isothermal amplification (LAMP).

LAMP technology is highly versatile and uniquely suited to use in resource-limited situations. The method utilises *Bacillus stearothermophilus* DNA polymerase *(Bst)* which allows the reaction to proceed isothermally, negating the requirement for thermocycling. *Bst* polymerase has also been shown to be more resistant to inhibitors than conventional

Thermus aquaticus (*taq*) polymerase, allowing amplification of relatively crude sample preparations (Edwards et al., 2014; Kaneko et al., 2007). Reaction components can be pre-mixed and lyophilized, making reaction preparation simple, therefore the test could be used with minimal training. For these reasons, LAMP assays have been widely developed for the detection of neglected tropical diseases including African sleeping sickness (Njiru et al., 2008), Loa Loa (Drame et al., 2014) and West Nile virus (Parida et al., 2004). LAMP assays have also been developed for a range of medical and veterinary diagnostics including *Escherichia coli* (Hill et al., 2008), *Plasmodium* species differentiation (Han et al., 2007), *Herpes simplex* virus (Enomoto et al., 2005), foot and mouth disease (Dukes et al., 2006) and orf virus (Tsai et al., 2009). Since LAMP reagents can be lyophilised, this technology has been utilised in the development of point of care (POC) devices from simple lateral flow sticks (Yongkiettrakul et al., 2017) to elaborate microfluidic chips capable of running multiple parallel reactions from a single sample (Fang et al., 2011). POC tests provide fast, personalised treatment decisions and could revolutionize human and veterinary medicine, particularly in cases of epidemics.

The majority of LAMP assays provide simple presence/absence results but quantification has been reported, based on time to amplification (Abbasi et al., 2016; Drame et al., 2014) or detection of an electrochemical signal monitoring the intercalation of DNA-binding reporter molecules (Hsieh et al., 2012).

Figure 1.9 illustrates the strand-displacement amplification method of LAMP. A set of 4-6 primers are used, two inner primers which bind first to initiate amplification and two outer primers which are required for the strand-displacement activity (Notomi et al., 2000), an additional pair of loop primers can be added to speed up the reaction under certain conditions (Nagamine et al., 2002). The inner primers (FIP and BIP) contain sequences complementary to both the sense (e.g. the F2 region of the FIP primer in Figure 1.9) and

antisense strands (e.g. F1c). The reaction is initiated by the inner primers and the binding of the outer primers then displaces the growing DNA strand. Once displaced, the antisense section of the inner primer binds to the complimentary downstream sequence producing the first loop structure (Figure 1.9). Several strand-displacement DNA amplification reactions progress in unison due to the sequential binding of the inner, outer and loop primers to the growing DNA molecules, building an array of complex stem-loop structures (Nagamine et al., 2002; Notomi et al., 2000). The detection of results can be tailored to the application of the assay, fluorescent, fluorochrome and colour change dyes are commonly included in the reaction mixture for immediate detection of results without downstream processing. Several studies have also reported the inclusion of intercalating reporter molecules or, alternatively, results can be monitored by measuring the turbidity of the reaction mixture as magnesium pyrophosphate builds up within the tube as a by-product of DNA amplification (Mori et al., 2001).

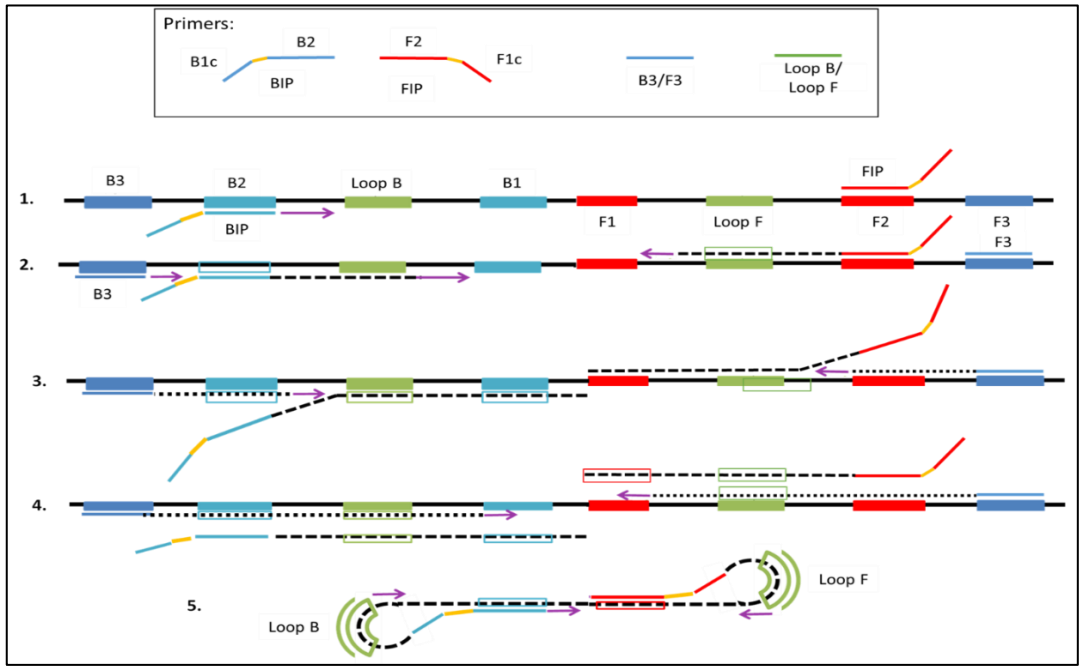


Figure 1.9. Diagram of the amplification method of Loop-mediated isothermal amplification (LAMP).

The LAMP primer set consists of two inner primers (FIP and BIP) which each comprise a sequence complimentary to the sense DNA strand (e.g. F2) and the antisense/reverse DNA strand (e.g. F1c) which are linked, two outer primers (F3 and B3) and sometimes two additional primers (loop F and loop B). The inner primers bind first to initiate amplification (1) followed by the outer primers (2). The first growing strand initiated by the inner primers is displaced by the second growing DNA strand (initiated by the outer primers) (3). Once displaced, the antisense section of the inner primer binds to the complimentary downstream sequence, producing the first loop structure (4/5). Several strand-displacement DNA amplification reactions progress in unison due to the sequential binding of the inner, outer and loop primers to the growing DNA molecules, building an array of complex stem-loop structures

1.8 Prevalence of BZ-resistance

1.8.1 Trichostrongylid species

The launch of the benzimidazole, levamisole and the macrocyclic lactones between 1961 and 1981 revolutionised GIN control. Heavy reliance on these compounds placed significant selection pressure on GIN populations which quickly led to the emergence of anthelmintic resistance. Table 1.3 summarises the emergence of anthelmintic resistance in each of the five main anthelmintic classes including the nematode species involved in the initial case of resistance. Resistance quickly emerged in other nematode species and multi-drug resistance (nematodes resistant to the more than one of the three broad-spectrum anthelmintics available at the time) was reported in the early 1980's (Conder and Campbell, 1995). Recent prevalence studies have found significant resistance to all three of the major anthelmintic classes (1-BZ, 2-LV and 3-ML) in the UK (Thomas et al., 2015) and throughout the world (Kaplan, 2004).

Table 1.3. Summary table of the five major anthelmintic classes.

The launch date, first report of anthelmintic resistance and the nematode species involved for each of the five major anthelmintic classes.

Drug	Year of Release	First Report of Resistance	Species involved in first case of resistance
Benzimidazole	1961	1964	*Haemonchus contortus* (Conway, 1964; Drudge et al., 1964)
Levamisole	1970	1979	*Teladorsagia circumcincta* (Le Jambre, 1979; Sangster et al., 1979)
Macrocyclic Lactones	1981	1988	*Haemonchus contortus* (van Wyk and Malan, 1988)
Amino-Acetonitrile Derivatives	2009	2013	*Teladorsagia circumcincta* & *Trichostrongylus colubriformis* (Scott et al., 2013)
Derquantel	2009	-	-

This thesis focuses on BZ-resistance which is particularly advanced. Figure 1.10 summarises the timeline of this drug class from release in 1961 to present day resistance status. The frequency of BZ-resistance varies between countries and GIN species with lower resistance observed in *Trichostrongylus* species compared to *T. circumcincta* and *H. contortus* (Ramunke et al., 2016).

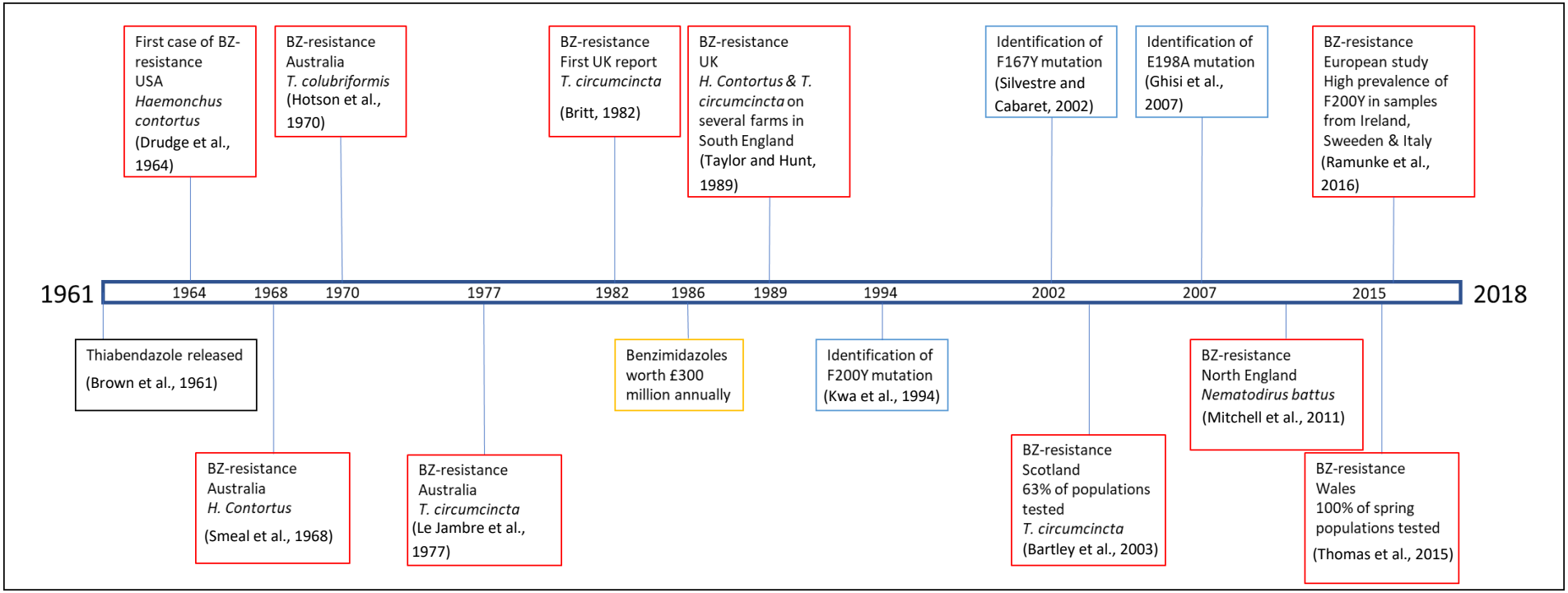


Figure 1.10. Timeline of benzimidazole resistance in ovine nematode species.

Red boxed text represents BZ-resistance, blue boxes indicate the discovery of SNPs associated with resistance and additional information is highlighted in yellow.

1.8.2 Nematodirus battus

Although anthelmintic resistance had not been reported in *N. battus* prior to 2010, BZ-resistance was confirmed in other *Nematodirus* species. BZ-resistance in *Nematodirus* was first suspected in 1982 (Jackson, 1982), confirmation was reported in 1983 with an oxfenbendazole-resistant *Nematodirus spathiger* isolate in New Zealand (Middelberg and McKenna, 1983). By 1984, several cases of BZ-resistant *N. spathiger* had been recorded throughout the North and South Islands of New Zealand (Vlassoff and Kettle, 1985) and later in Southern Australia (Beveridge et al., 1990; Obendorf et al., 1991). *N. spathiger* is the predominant *Nematodirus* species causing significant disease in the southern hemisphere with low level co-infection with *Nematodirus filicollis* (Obendorf et al., 1986), *N. battus* has not been identified in Australia or New Zealand. BZ-resistance has also been confirmed in *N. filicollis*, originally in Australia (Beveridge et al., 1990) and more recently in New Zealand (Oliver et al., 2016a). The current prevalence of BZ-resistance in New Zealand *Nematodirus* populations was found to be significant with around 95% in *N. spathiger* and 40% in *N. filicollis* populations tested showing <95% reduction in FEC following treatment (Oliver et al., 2016a). Few other reports of BZ-resistance have been published to date out-with Australasia (Bentounsi et al., 2007; Diez-Banos et al., 2008; Mohamed and Al-Farwachi, 2008; Rosalinski-Moraes et al., 2007) and no information is available regarding the prevalence of BZ-resistance in *N. battus* populations.

1.9 Aims of the project:

The epidemiology of *N. battus* has changed in recent years with the emergence of anthelmintic resistance and varied patterns of detection and disease being reported in the field. This project aimed to address the knowledge gaps in our current understanding of *N. battus* behaviour and the changes being observing. BZ-resistance was only identified in this species for the first time in 2010 despite heavy reliance on benzimidazoles to control *N. battus* for over 50 years. It was therefore vital to understand more about resistance in this species to inform effective control. As discussed, FECRT remains the gold-standard on-farm method of detecting anthelmintic resistance. However, this method is not ideal for diagnosing resistance in *N. battus* due to the rapid expulsion of adult parasites during acute infections. The time and labour required to re-gather and sample animals post treatment may also be a barrier to uptake. The first aim of the project was to develop and evaluate molecular tools for the detection and quantification of the SNPs in the β-tubulin isotype 1 gene associated with BZ-resistance in *N. battus*. Both laboratory-based platforms and pen-side applications were explored during the project using pyrosequencing, Illumina MiSeq next generation sequencing and loop mediated isothermal amplification (LAMP). The second aim was to use the developed tools to assess the prevalence of BZ-resistance in *N. battus* populations throughout the UK. Compared to BZ-resistance in other GIN species, resistance in *N. battus* appeared to be slow to develop. Despite several factors having been associated with the development of anthelmintic resistance in other nematode species, it remains unclear whether the same factors would influence the development of resistance in this species. Differences in the epidemiology and life history traits between *N. battus* and other GIN species may influence the development and dissemination of anthelmintic resistance. The third aim was to address the lack of knowledge relating to *N. battus*. Detailed information on current farm management and parasite control strategies in use throughout the UK was gathered using an online questionnaire distributed to both study farms involved

in the genotyping survey and to the wider farming community. The information gathered was used to explore potential management and environmental risk factors associated with BZ-resistance in *N. battus* using generalised linear mixed effect modelling. As well as the emergence of resistance in the species, the epidemiology of *N. battus* also appeared to be changing with increasing reports of infection throughout the year, particularly in autumn. Previous research highlighted that some *N. battus* eggs did not require a period of chilling as was previously believed to be a requirement for successful hatching. Although egg hatching without chilling was known to be possible, it had not been quantified and the factors governing the requirement for chilling in *N. battus* eggs had not been studied. With increasing uncertainty in the timing of hatching in this species as a result of climate change, online risk maps predicting risk periods for hatching are becoming more commonly used. The models driving the risk maps are based on the hatching of chilled eggs and do not account for un-chilled egg hatching therefore farmers could be at risk of unexpected disease outbreaks. The fourth aim of this project was to quantify non-chill hatching in *N. battus* eggs. To explore the prevalence of this phenomenon both within and between populations and create a picture of the incidence of non-chill hatching in populations collected from commercial farms throughout the country. Finally, the project also aimed to explore the management and environmental factors associated with non-chill hatching in *N. battus* eggs in order to better understand the hatching behaviour of this species, which is key to the development of control strategies. *N. battus* remains an economically important parasite in the UK which threatens the health and welfare of lambs each year. Overall the project aimed to develop tools to enable the study of this species, to provide an understanding of the current epidemiology and anthelmintic resistance status in populations collected from UK commercial farms and to identify potential drivers influencing the novel changes observed. The findings of the project may be used to update advice and risk maps and to inform the development of future control strategies.

2 Prevalence of SNPs associated with benzimidazole resistance in UK *N. battus* populations

2.1 Abstract

Benzimidazole compounds are heavily relied upon for the control of *Nematodirus battus* on UK sheep farms. The first case of BZ-resistance in this species was identified in 2010 however, the extent of resistance in this species has not previously been determined. The current genotyping study assessed the prevalence of single nucleotide polymorphism (SNP) mutations at codons F167Y, E198A and F200Y within the β-tubulin isotype 1 gene which are widely associated with BZ-resistance in this and other ovine nematode species. A total of 273 populations from 248 farms were successfully analysed by pyrosequencing; 30 individual nematode eggs or infective larvae (L_3) were genotyped per population. F200Y was found to be the most prevalent SNP, identified in around one in four of the populations tested with a low overall frequency of 2.1±0.6% (mean % ± SEM). F200Y alleles were found to be widespread throughout the UK however, a focal region of high resistant allele frequency was identified in the area surrounding the initial BZ-resistant *N. battus* isolate. The F167Y SNP was also identified in a small number of populations (four isolates) at a low allele frequency (1.3% ±0.01). The F167Y and F200Y SNPs were found to be mutually exclusive and F167Y was identified independent of F200Y in one isolate. E198A was not identified in any of the isolates tested. The current study has created a benchmark for BZ-resistance in *N. battus* from which the progression of anthelmintic resistance in the species can be measured. The high prevalence of resistant alleles in the *N. battus* populations tested, albeit at low allele frequency highlights the potential for expansion in the future.

2.2 Introduction

Prior to confirmatory findings in 2010 (Mitchell et al., 2011), *Nematodirus battus* was believed to be refractory to the development of benzimidazole resistance. This perception was driven partly by the lack of any reports of resistance within this species in the face of over 50 years of usage in sheep around the world but also because benzimidazole resistance had been confirmed in the closely relate *Nematodirus* species *N. spathiger* (Chalmers, 1985; Little et al., 2010; Macchi et al., 2001; McKenna et al., 1995; Middelberg and McKenna, 1983; Vlassoff and Kettle, 1985) and *N. filicollis* (Beveridge et al., 1990; Oliver et al., 2016a) and was estimated to be at a significant levels in New Zealand in 2016 (Oliver et al., 2016a). Information regarding the extent and distribution of resistant alleles within a population is essential for the development of effective control strategies. Characterisation of the initial BZ-resistant *N. battus* isolate identified the F200Y single nucleotide polymorphisms (SNP) in β-tubulin isotype 1 gene (Morrison et al., 2014), previously associated with BZ-resistance in other GIN species (Elard and Humbert, 1999; Kwa et al., 1994) and suggested that this SNP may also confer resistance in *N. battus*.

Pyrosequencing is a well-established technique for the detection of SNPs (Morrison et al., 2014; Ramunke et al., 2016; von Samson-Himmelstjerna et al., 2009), and has been widely utilised in genotype prevalence studies worldwide (Chaudhry et al., 2015b; Ramunke et al., 2016; Redman et al., 2015). Morrison et al previously published a pyrosequencing assay for the detection of F200Y SNP in *N. battus* (Morrison et al., 2014), which provides detailed information from the analysis of individual parasites.

The aims of the current study were to explore the distribution of *N. battus* throughout the UK, to create an accurate picture of the prevalence of F200Y at the individual and population level on farms, exploring the regional distribution of resistant alleles, to investigate the presence of other SNP mutations associated with benzimidazole resistance in UK *N. battus*

populations, to provide information to inform recommendations on the use of benzimidazoles to control *N. battus* and to offer a baseline measurement for future comparisons.

2.3 Methods

2.3.1 Sample collection

A total of 381 *N. battus* populations from 348 farms were collected between 2011 and 2016 and were included in this study as detailed in figure 2.1.

Populations were collected in a non-stratified independent fashion but attempts were made to balance for perceived spatial bias. A number of samples were submitted by Animal and Plant Health Agency (APHA) and Scotland's Rural College (SRUC) surveillance centres from across the UK. Additional samples were collected opportunistically in 2015 in a non-random fashion. Farm visits in 2015 included farms from the local region surrounding the initial case of BZ-resistance in this species. Sampling in 2016 was targeted to regions which were under-represented in the biobank of *N. battus* isolates collected, but which appeared to have significant sheep densities (sheep density data from the Office for National Statistics in 2009 was mapped using QGIS; data source Geo-wiki). Farms in the target regions were contacted via local advisors, veterinarians and the Animal and Horticulture Development Board (AHDB). 2016 sampling was focused in South West England and farm visits were conducted in North East Scotland and the Scottish borders to increase sample numbers in these regions. Although sampling bias may have been introduced in regions visited by the research team during sampling trips, the aim of the study was to explore the presence of BZ-resistance alleles in UK *N. battus* populations and to investigate risk factors associated, not to conduct a stratified prevalence survey. Sampling trips lasted 1-2 days therefore storage of samples collected by this method was of similar duration and conditions as those submitted by post.

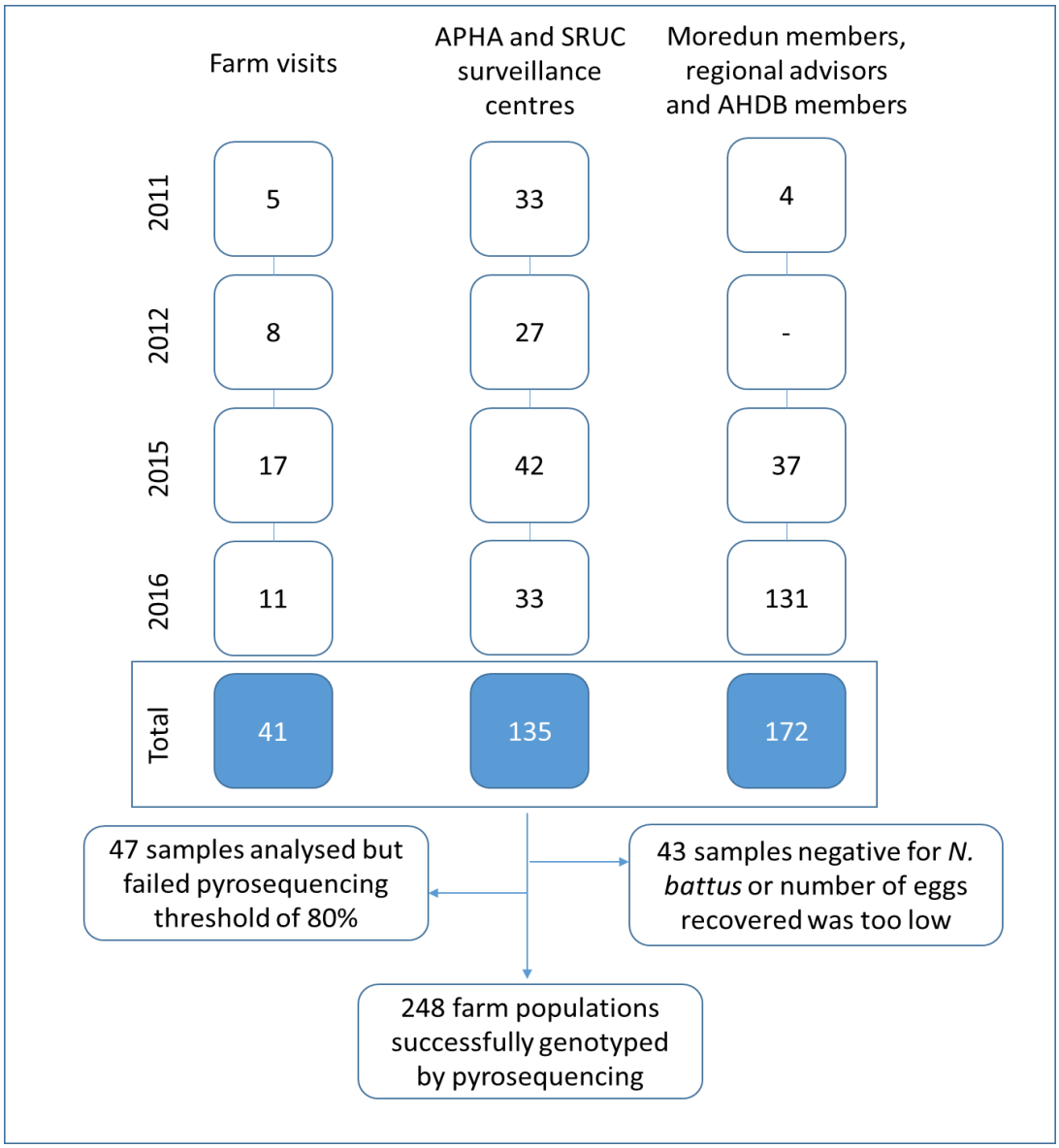


Figure 2.1. Flow diagram of how many farm samples were collected from surveillance centres, AHDB and Moredun members and in-person farm visits during each sampling year.

During farm visits, fresh lamb faecal samples were collected from the ground, at least 10 per farm or field. Faecal samples were placed in plastic bags, excess air removed, and stored at 4°C once returned to the laboratory to suspend development prior to processing. Samples which were submitted by SRUC and APHA surveillance centres were packaged in air-tight containers for postage. Sample collection packs were posted to farmers who volunteered to

submit samples for the present study. Each pack contained instructions on sample collection, 10 plastic zip lock bags, gloves and a freepost return envelope. Farmers were instructed to collect 10 fresh lamb faecal samples from the ground, sealing each in an individual zip lock bag with excess air removed, samples were then packaged following royal mail guidelines for biological samples and posted back to the laboratory where samples were stored at 4˚C upon arrival.

2.3.2 Sample preparation and DNA extraction

Faecal egg counts (Jackson and Christie, 1972) were conducted on all samples to confirm the presence of *N. battus* eggs prior to egg extraction, samples were stored at 4°C for up to 5 days before processing.

Eggs were extracted from faeces by differential sieving. Briefly, individual faecal samples were pooled by field or farm, homogenised in tap water and thoroughly washed over stacked sieves; 212µm, 125µm and 53µm. *N. battus* eggs and fine faecal debris collected on the 53µm sieve were washed into 12ml polyallomer tubes (Beckman Coulter Inc., USA) to clean the filtrate. Samples were centrifuged at 203 x g for 2 minutes, the supernatant subsequently removed and 10ml of saturated sodium chloride solution (specific gravity 1.2) added. Tubes were inverted to disrupt and re-suspend the faecal pellet prior to centrifugation (203 x g, 2 minutes), *N. battus* eggs float in saturated NaCl solution and were therefore isolated from the faecal debris. Artery forceps were used to clip the top of each tube, isolating the eggs in the top segment of liquid. Eggs were poured over a 53µm sieve and washed with excess tap water to remove remaining salt and placed into non-air-tight jars with tap water, one culture per field or farm population. Egg cultures were stored at ambient room temperature, protected from direct sunlight to allow for larval development. Cultures were monitored microscopically for development and hatching (Figure 2.2). Larvated eggs and third stage

larvae (L_3) were concentrated and fixed in ethanol (final concentration >70% EtOH) prior to molecular analysis.

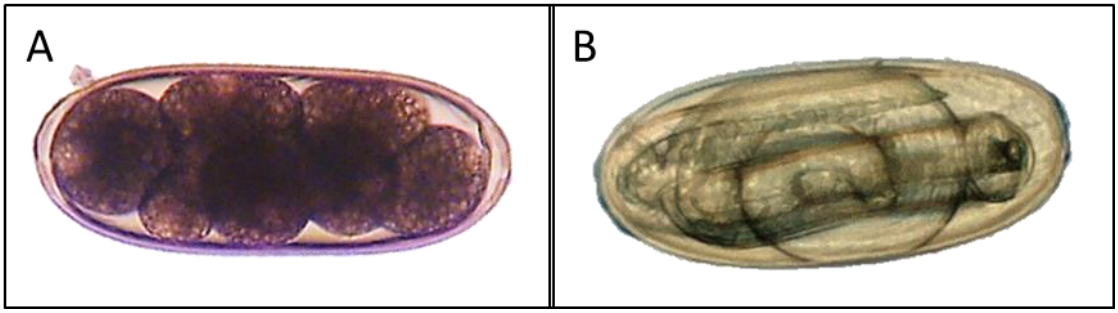


Figure 2.2. N. battus eggs at different development stages.

N. battus eggs (a) pre-developed and (b) embryonated stage, L_3 visible inside the egg.

Ethanol-fixed eggs and larvae were re-suspended in 1X phosphate-buffered solution (PBS) (1:100 v/v) for 30 minutes to rehydrate. Thirty individual parasites from each farm population were picked at random in 1µl into individual wells of a 96 well plate (Axygen, USA), containing 15µl lysis buffer (50mM KCl, 2.5mM $MgCl_2$, 10mM Tris (pH 8.3) 0.45% Nonidet P-40, 0.45% Tween 20, 0.01% Gelatine) (Kwa et al., 1995). As parasite cultures were pooled by field or farm, eggs/L_3 selected for genotyping were representative of the population rather than any individual animal. Samples containing eggs were then subjected to 8 cycles of 30 second freeze in liquid nitrogen followed by one minute incubation at 100°C to weaken the egg shells. A further 15 µl of worm lysis buffer containing 0.2 mg/ml proteinase K was added to each well of the plate for both egg and larvae samples. Plates were incubation at 56°C overnight, the temperature was increased to 92°C for 10 minutes to deactivate the proteinase K. Crude lysates were used directly as template in PCR reactions.

2.3.3 DNA amplification and pyrosequencing

The *N. battus*-specific 198/200 SNP assay used was previously detailed by Morison *et al.* (2014), PCR and pyrosequencing primers for analysis of the 167 SNP are detailed in table 1. PCR reactions for 198/200 and 167 were conducted using NovaTaq Hot start master mix (Merck, USA) in 50µl volumes containing 0.185µM reverse primer, 0.2µM biotinylated forward primer, 4.5mM $MgCl_2$, 25µl 2 x buffer and 4µl of template DNA. PCR reactions were incubated at 95°C for 10 minutes followed by 45 cycles at 94°C for 30s, anneal for 30s at 58°C for the P198/200 assay or 54°C for P167, and 72°C for 30s with a final extension phase at 72°C for 10 minutes. DNA amplification was confirmed by gel electrophoresis; 5 µl of PCR product was run on a 2% agarose gel stained with gel red (Biotium, USA). Provided DNA amplification was confirmed, the remaining PCR product was analysed using the *N. battus*-specific 198/P200 pyrosequencing assay (Pyromark ID, Qiagen, Germany) previously described by Morrison *et al.* (2014). A total of five negative controls were included per 96-well plate for pyrosequencing to test for contamination during DNA preparation and analysis. When analysing pyrosequencing results, populations in which fewer than 80% of the individual egg/L_3 DNA lysates were successfully genotyped were removed from downstream analysis.

A subset of populations were selected for 167 analysis based on the results of Illumina MiSeq analysis, detailed in chapter 4. DNA plates prepared for F200Y analysis were re-used for F167Y testing. Table 1 details the primers used for F167Y analysis, the pyrosequencing protocol was the same as that used for F200Y analysis.

Table 2.1. PCR and sequencing primers for genotyping the F167Y SNP in N. battus by pyrosequencing.

Primer	**5' – 3'**
Forward (biotinylated)	CGTGAGGAGTACCCCGATAGGA
Reverse	AGTTCGGGATTTAACGAAGAGC
Sequencing	GGCGACGGAACGACA

2.3.4 Statistical analysis

Populations were divided into regions for analysis based on the map boundaries detailed in Figure 2.3. Binomial logistic regression analysis was carried out to calculate the increase in risk of identifying F200Y resistant alleles between regions using pyrosequencing results. Analysis was carried out using R version 3.2.5. Hardy-Weinberg analysis was performed to determine whether the loci were under active selection at the point of the study. Observed and expected homo/hetero-zygote frequencies were compared using a chi-squared analysis, performed in Microsoft Excel.

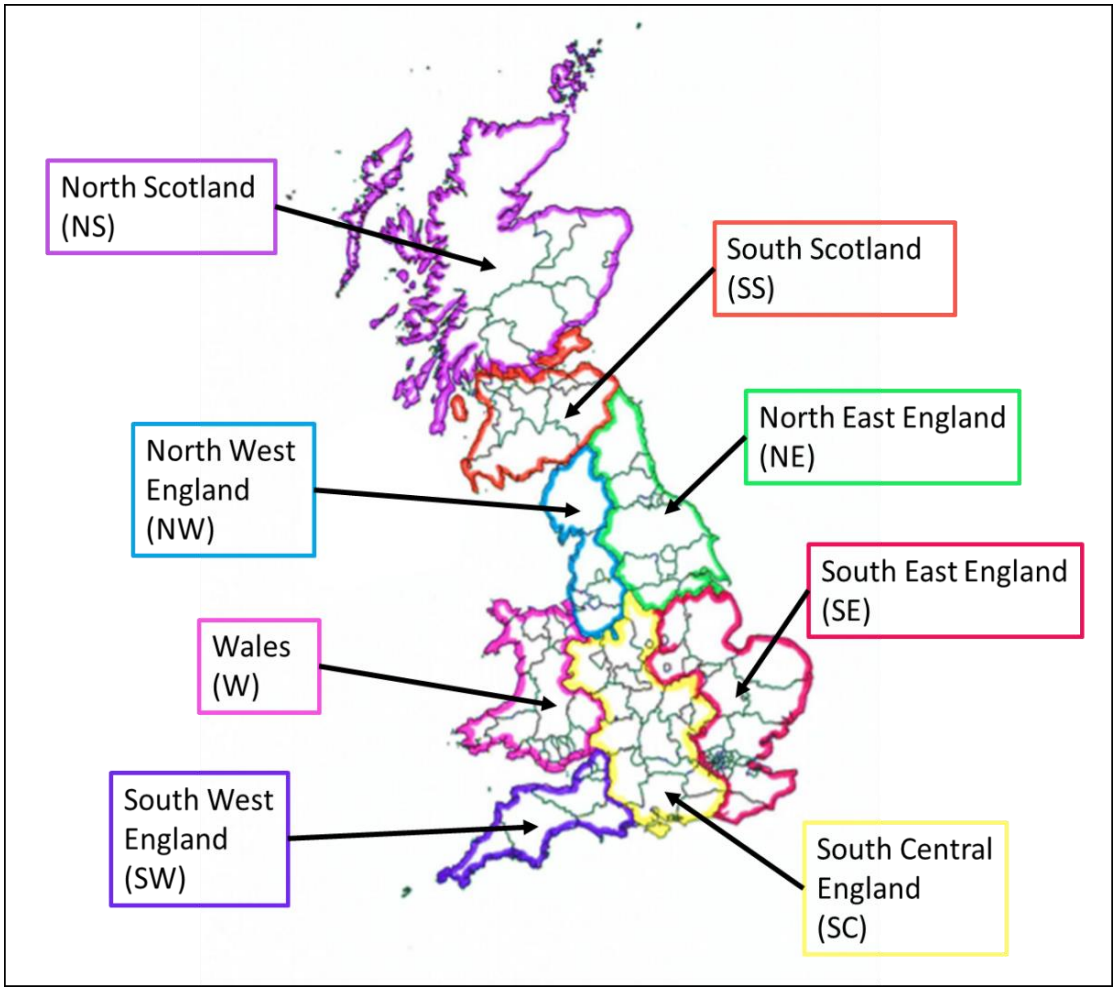


Figure 2.3. Map of UK regional boundaries used to divide sample populations for data analysis throughout the project.

2.4 Results

2.4.1 Faecal egg counts

A total of 2595 faecal samples from 348 farms were collected and processed from throughout the UK in 2011 to 2016. Of the farms tested, 94% (n = 329) were positive for *N. battus* with the overall average faecal egg count of 161 eggs per gram (EPG), individual positive counts ranged from 1 to 3330 EPG. Strongyle eggs were also counted; average 141 EPG, range 0 – 2322 EPG. Figure 2.4 and Figure 2.5 show individual faecal egg count results of samples collected from 308 of the farms included in the study. The graphs highlight the variation observed in both *N. battus* and strongyle egg count both within and between populations. Despite the variation observed at the individual and population level, Figure 2.6 shows little difference in *N. battus* EPG between geographic regions of the UK.

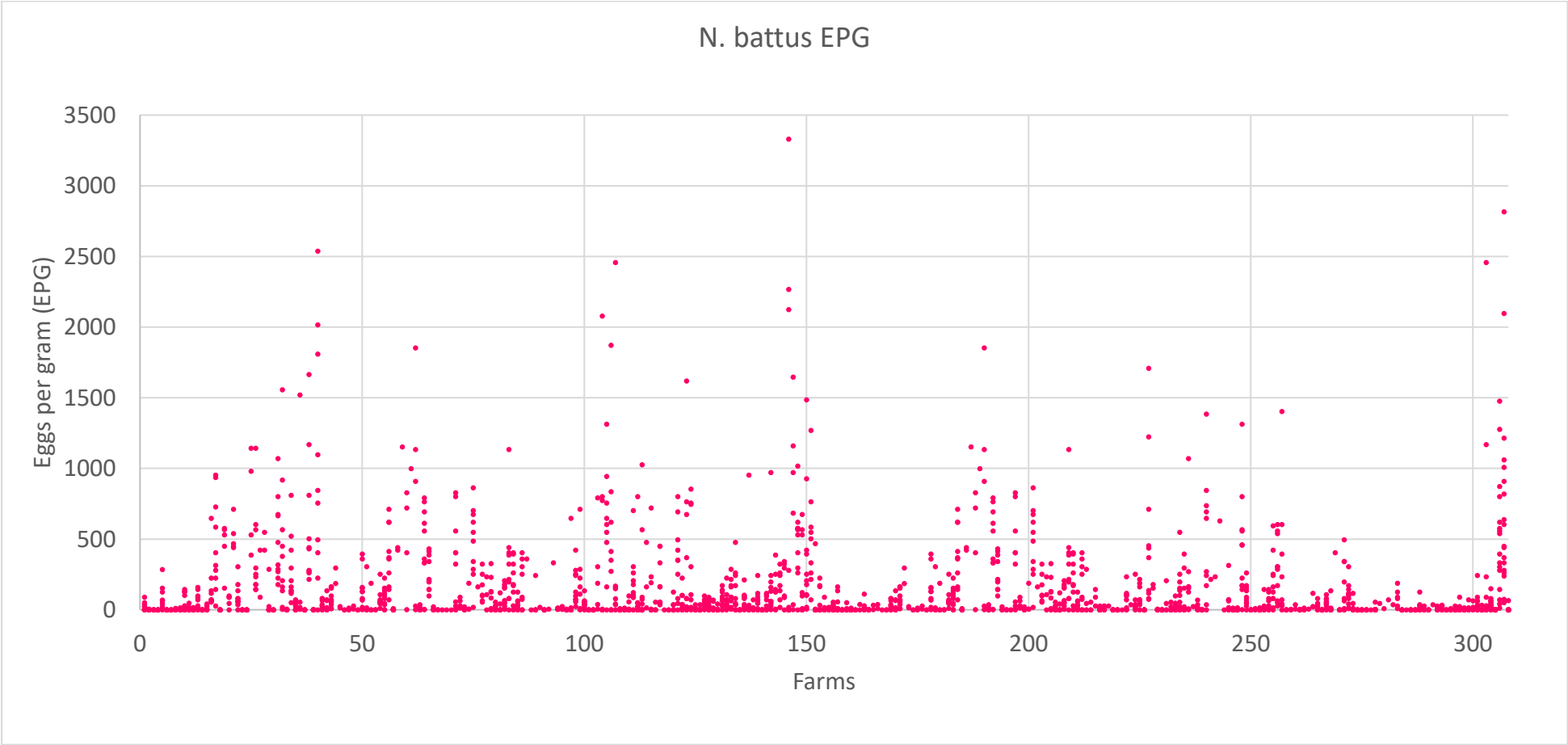


Figure 2.4. Nematodirus faecal egg count results.

Individual faecal egg counts from samples collected from 308 of the farms included in the project.

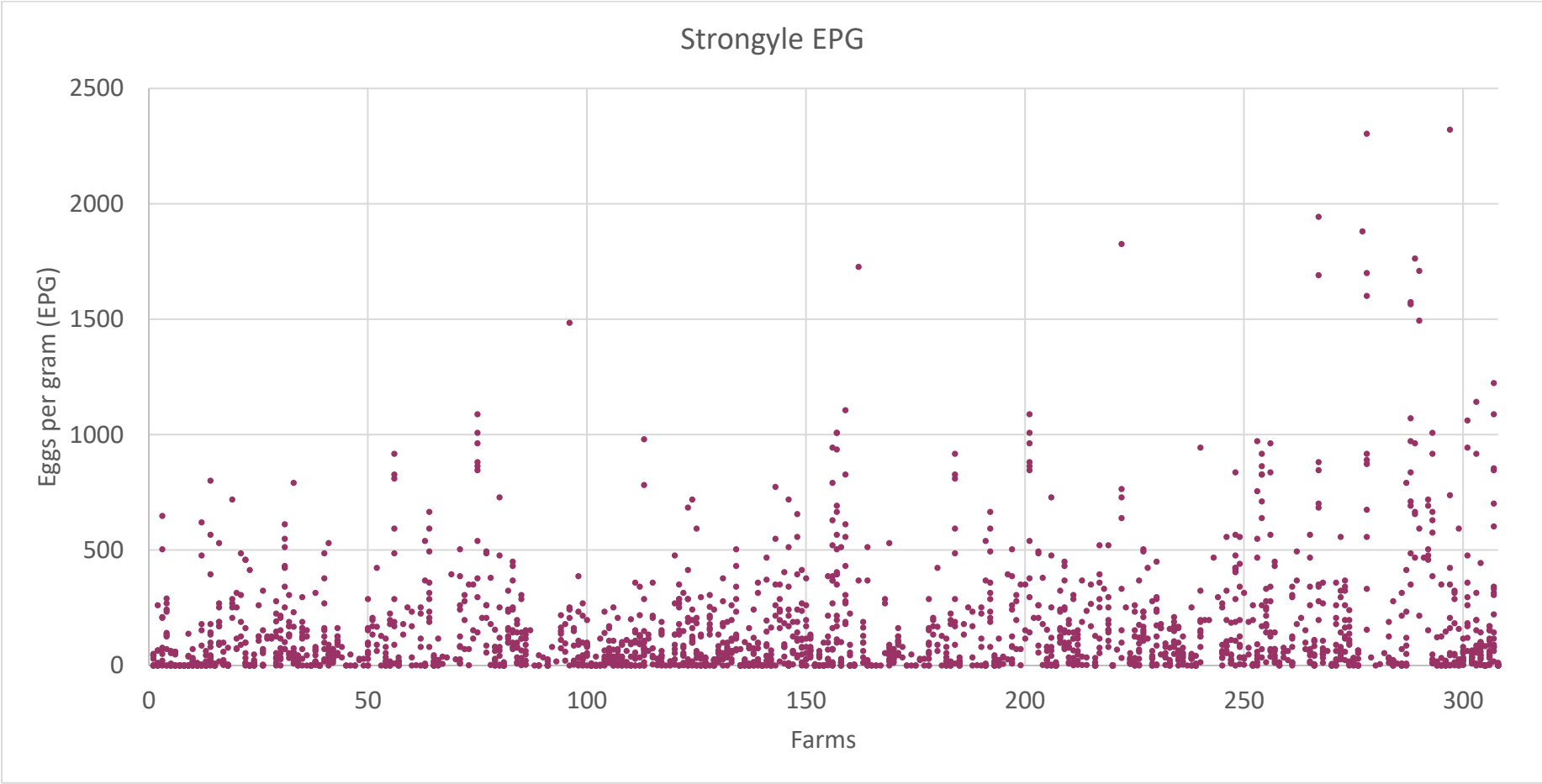


Figure 2.5. Strongyle faecal egg count results.

Individual faecal egg counts from samples collected from 308 of the farms included in the project.

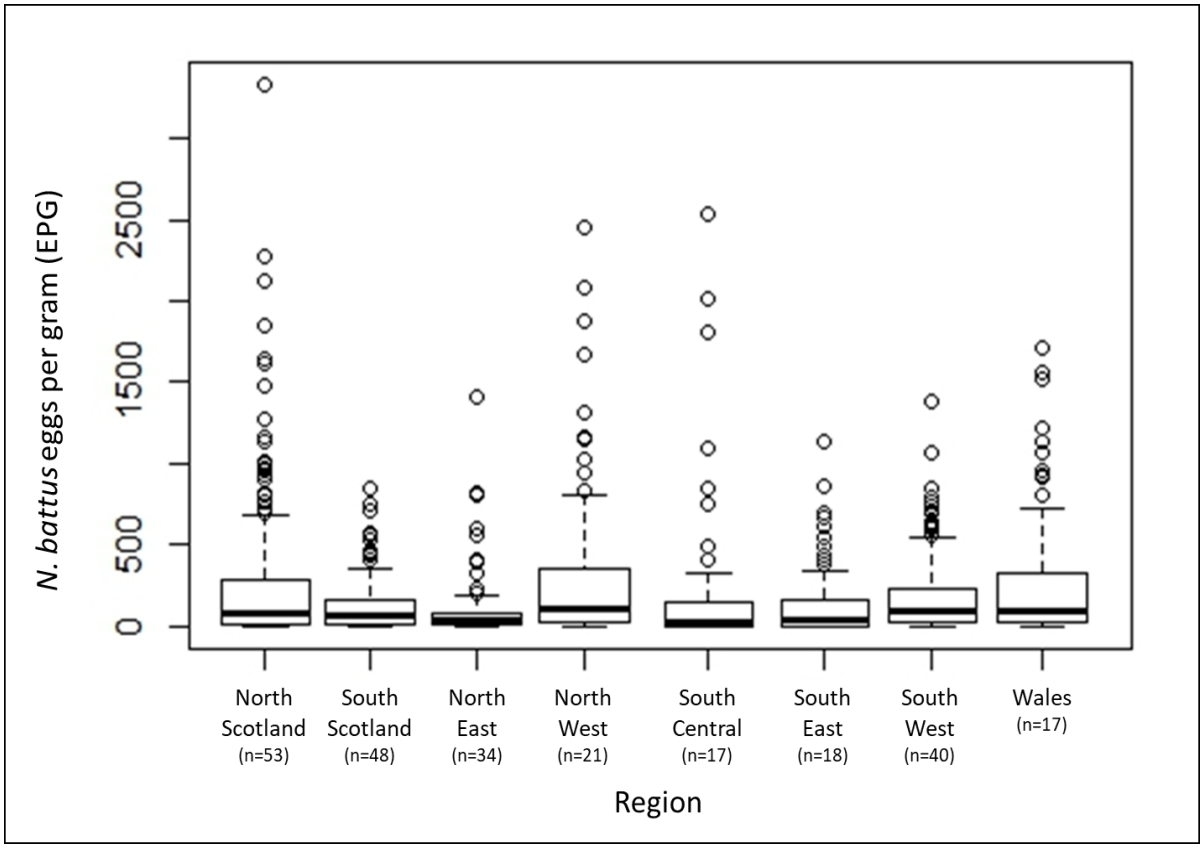


Figure 2.6. Boxplot of individual N. battus faecal egg count per UK region.

Regional faecal egg count results from individual samples with number of sample populations per region in brackets. The bold line highlights the median and the box, the upper and lower quartiles. Graph represents only those populations which were retained in the genotyping survey analysis.

2.4.2 Prevalence of F167Y in UK *N. battus* populations

A total of 18 populations from 17 farms were successful analysed at position 167. The resistant allele F167Y was identified in four populations at a low frequency; 1.3 ±0.01% (mean ±SEM), range, 0-13% (Figure 2.7).

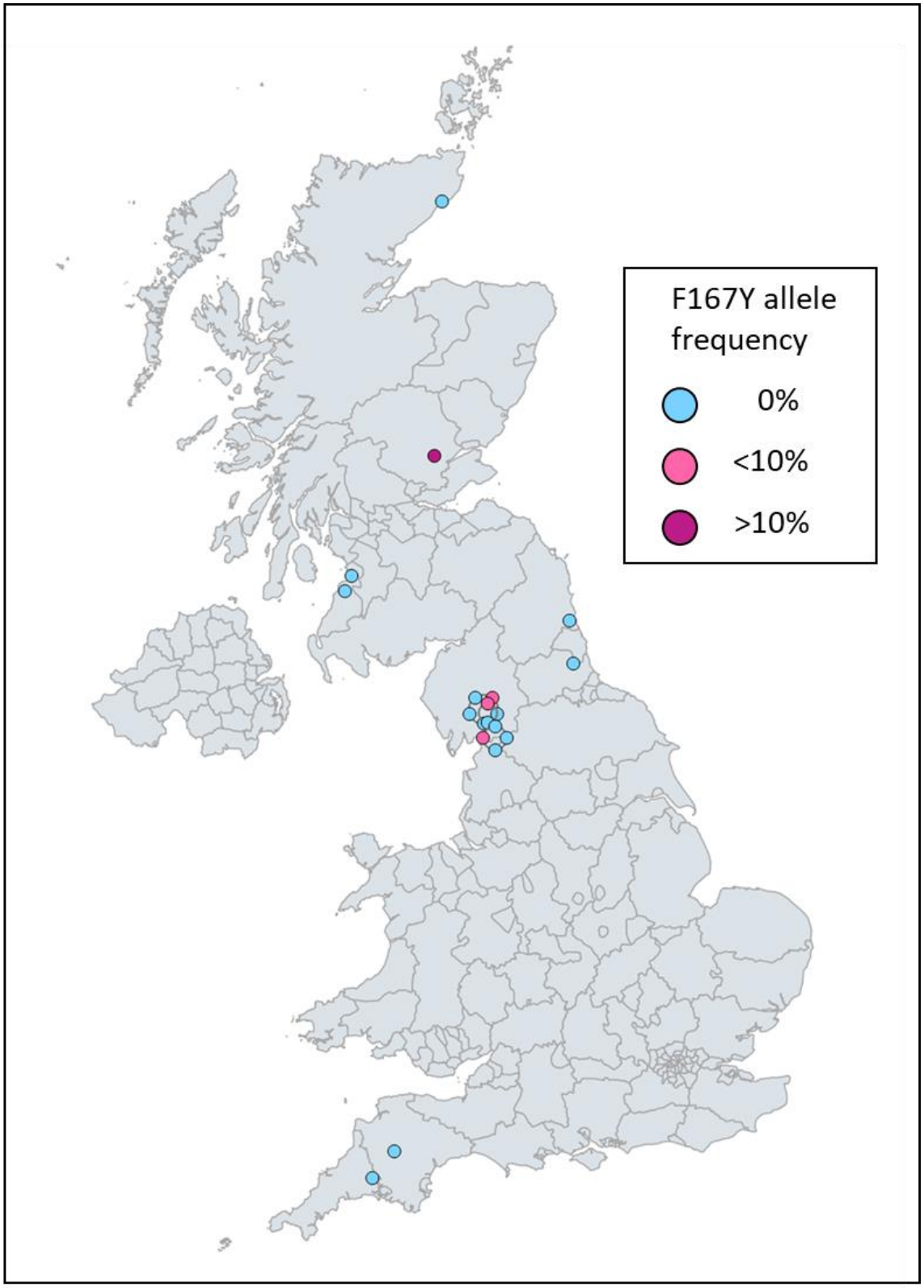


Figure 2.7. Map of the distribution of N. battus isolates in which the F167Y allele was identified.

The colour of the markers represents the F167Y allele frequency; no mutation at codon 167 identified (blue), <5% F167Y (pink) or >5% F167Y (purple).

2.4.3 Prevalence of E198A in UK *N. battus* populations

A total of 273 samples from 248 farms were analysed at codon position 198 however, the SNP was not identified in any samples tested.

2.4.4 Prevalence of F200Y in UK *N. battus* populations

A total of 273 samples from 248 farms were successful analysed at codon position 200. The F200Y mutation was identified on 65 farm populations throughout the UK with a low overall frequency; (mean ±SEM) 2.1±0.6% (Table 2.2), a total of eight populations (3%) were found to have a resistant allele frequency greater than 10%. The resistant allele was identified at a frequency of 2.9±0.9% in England and 1.1±0.7% and 1.7±0.6% in Scotland and Wales respectively. Despite the low overall frequency, the F200Y mutation was detected at a relatively high prevalence; in 26% of the populations tested.

Table 2.2. Mean regional F200Y allele frequency identified by pyrosequencing in Scottish, English and Welsh N. battus farm populations.

Country	Region	n	n with r-allele	%SS	%Sr	%rr	F200Y allele frequency	range
All		248	65	97.0	1.5	1.5	2.1	0-93
Scotland	Overall	101	16	97.8	1.0	1.2	1.7	0-65
	North	53	10	97.8	1.0	1.2	1.7	0-65
	South	48	6	99.4	0.3	0.3	0.4	0-4
England	Overall	130	41	95.8	2.1	2.1	2.9	0-93
	North East	34	10	98.4	1.0	0.6	1.1	0-6
	North West	21	12	81.8	8.1	10.2	12.6	0-93
	South central	17	4	98.7	0.9	0.5	0.9	0-8
	South East	18	3	98.5	0.9	0.7	1.1	0-13
	South West	40	12	98.4	1.0	0.6	1.1	0-6
Wales		17	8	97.5	1.6	0.9	1.7	0-8

The F200Y resistant allele was widely distributed UK with apparent 'focal regions' in which several local populations possessed higher than average resistant allele frequency (Figure 2.8). Logistic regression analysis of the distribution of resistant alleles identified that F200Y was almost 3 times more likely in North West England; the location of the initial case of benzimidazole resistance in this species; compared to other regions of the UK (estimate 2.7; 95% CI. 2.1 - 3.5, $p<0.001$). The resistant allele frequency was found to be lower in Southern Scotland compared to the UK average however, this difference was not statistically significant by logistic regression (estimate -0.9; 95% CI. -1.8, 0.1; $p=0.069$).

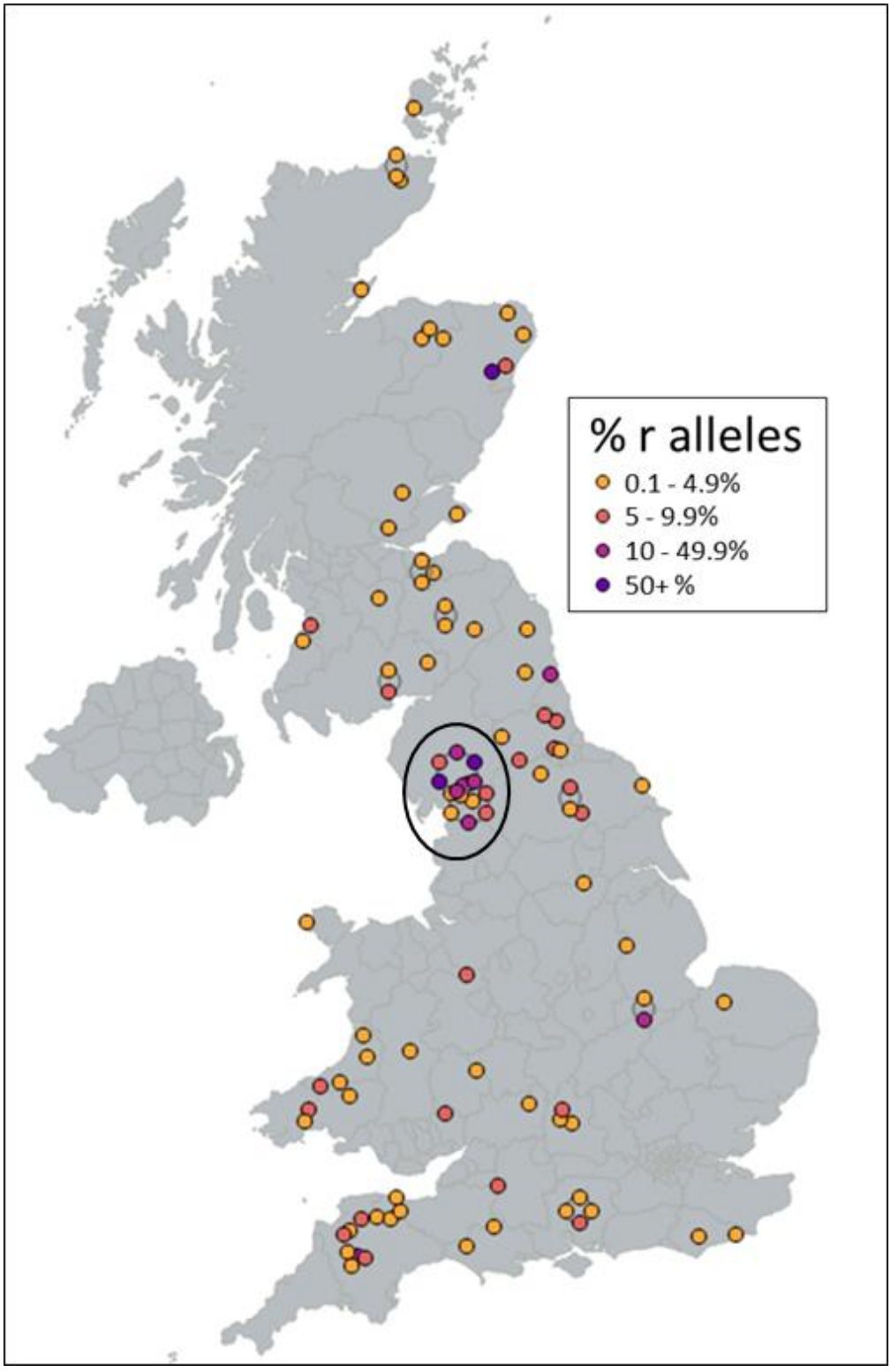


Figure 2.8. Map of the distribution of N. battus isolates in which the F200Y allele was identified.

The colour of the markers represents the F200Y allele frequency; 0.1-4.9% F200Y (yellow), 5-9% F200Y alleles (orange), 10-49.9% F200Y alleles (pink), >50% F200Y (purple). Circled is the focal region of high F200Y resistant allele frequency identified by binomial logistic regression analysis.

2.4.5 Analysis of genotype profiles over time: repeated samples

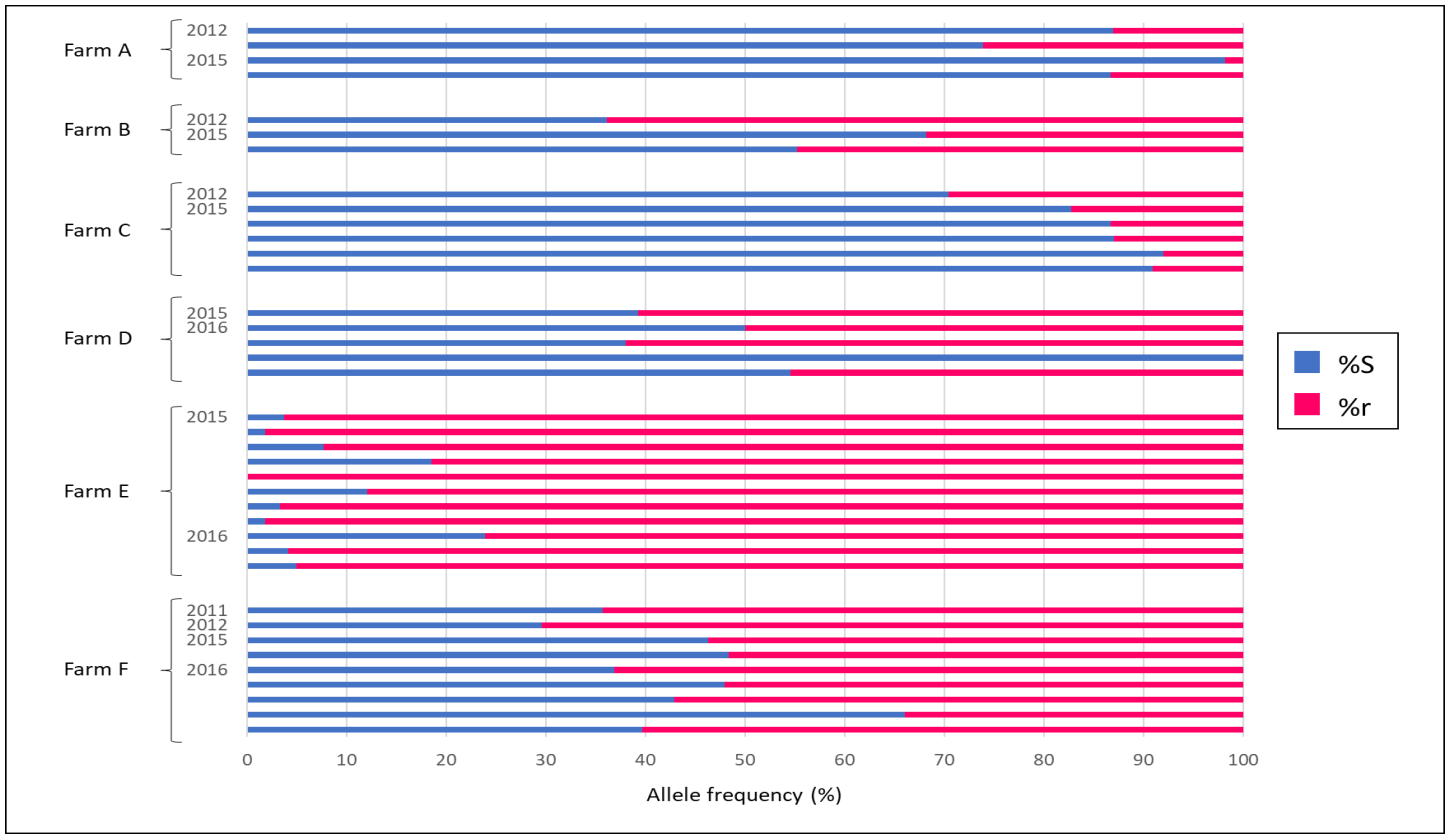


Figure 2.9. F200Y resistant allele frequency of repeated samples.

F200Y genotyping results (susceptible (TAC) allele frequency in blue and resistant (TTC) in pink) from repeated samples collected from six farms located in North West England between 2011 and 2016.

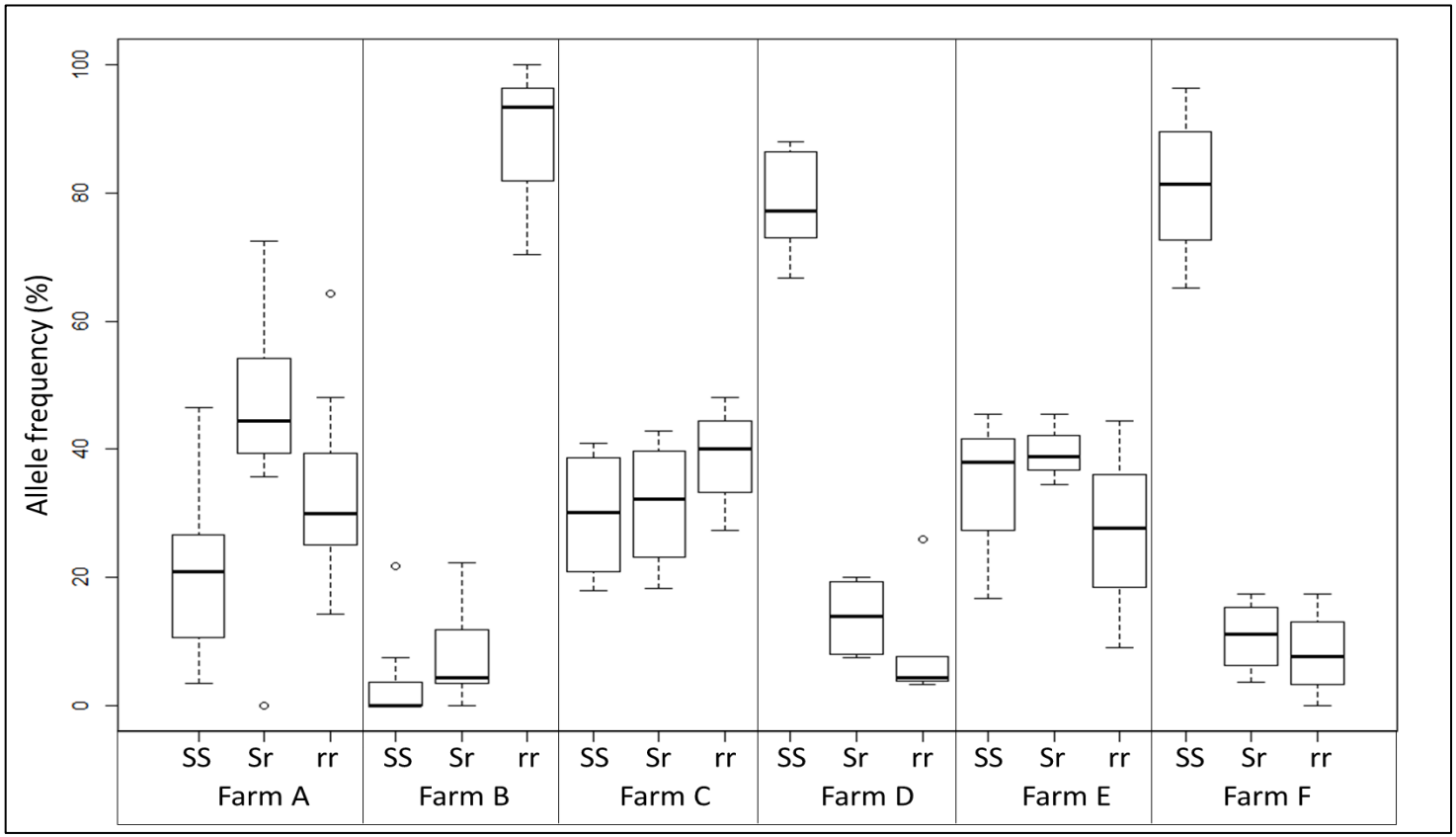


Figure 2.10. F200Y genotype frequencies obtained from repeated sampling.

Boxplot indicating the variation in genotype frequencies (homozygous susceptible (SS), heterozygous (Sr) and homozygous resistant (rr)) of repeated samples collected from 6 farms within the focal region of BZ-resistance from 2011 to 2016.

A subset of six farms from North West England were repeatedly sampled between 2011 and 2016 to assess the progression of BZ-resistance over time. Resistant allele frequency (F200Y) varied between samples however remained relatively stable over time (Figure 2.9). The results obtained from farm C showed a lower overall resistant allele frequency in 2015 compared to 2012, perhaps the result of altered management practices following the observation of resistant alleles within the *N. battus* population. The profile of heterozygous/homozygous frequencies also showed a small amount of variation across the timepoints (Figure 2.10) however, the overall genotype remained fairly constant over time.

2.4.6 Mutual exclusivity of F200Y and F167Y

The F200Y and F167Y SNPs were found to be mutually exclusive, i.e. an individual parasite could be heterozygous for both mutations but individuals which were homozygous resistant at either position were homozygous susceptible at the other. Figure 2.11displays the F200Y and F167Y genotype profiles of the 19 farms sequenced at both codons. The mutation at codon 200 was identified in 16 of the populations whereas F167Y was present in only four. F167Y was identified in three populations from North West England which possessed a significant F200Y allele frequency and a further population in North Scotland in which F167Y was present at a frequency of 13% with no F200Y resistant alleles identified.

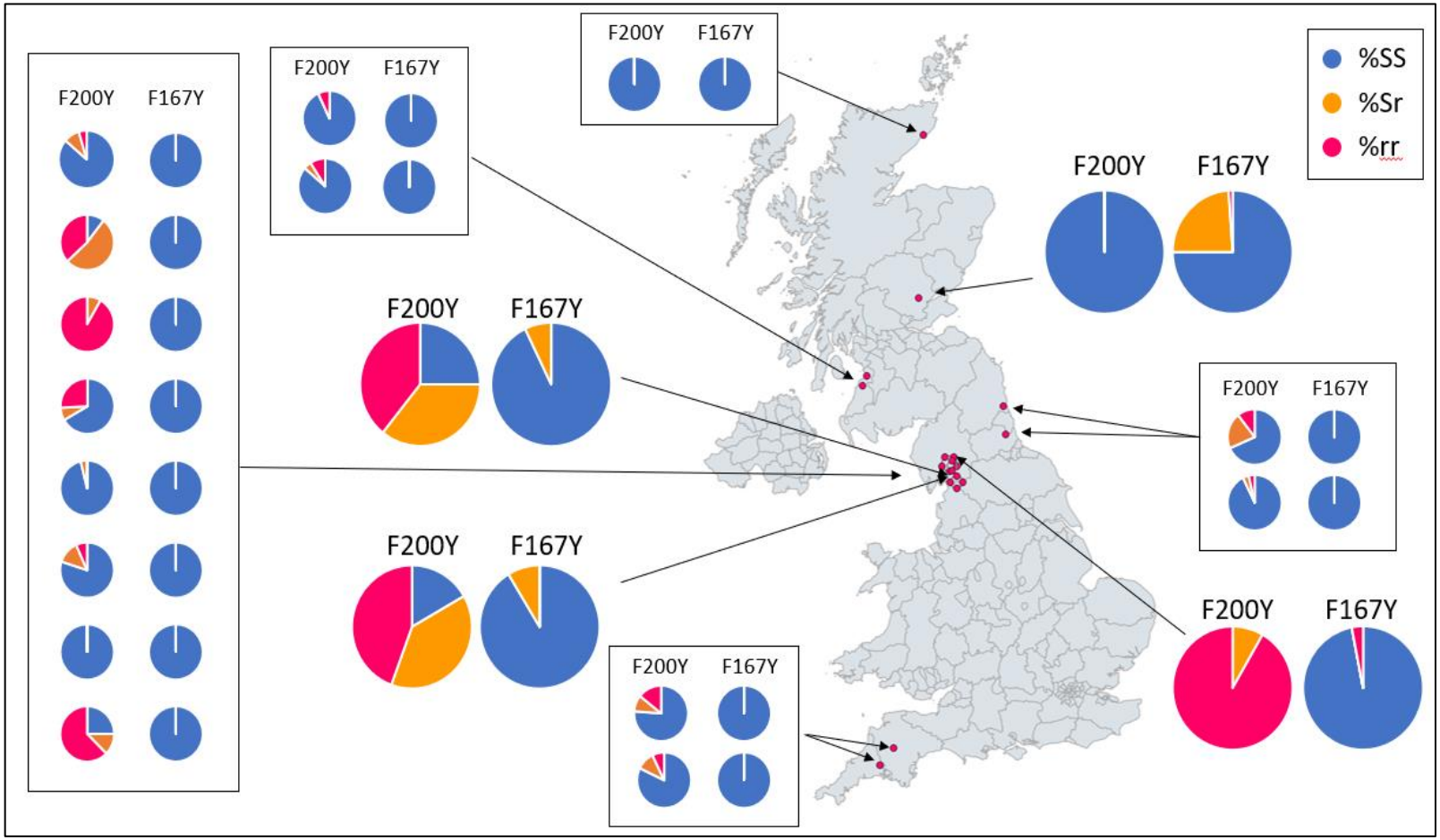


Figure 2.11. Genotype profile (% homozygous susceptible (SS), heterozygous (Sr) and homozygous resistant (rr)) of the F200Y and F167Y codons of the 19 farms analysed at both loci by pyrosequencing.

2.4.7 Hardy-Weinberg analysis

The heterozygote: homozygote ratios observed in the study populations at codons 167 and 200 were compared with the Hardy-Weinberg equilibrium to determine whether the loci were under active selection at the time of sampling. The allele frequencies observed at position 167 were not significantly different from that which would be expected given the Hardy-Weinberg equilibrium (χ^2=0.06, p=1), suggesting that the locus is not under selection. However, analysis concluded that codon 200 was under active selection in *N. battus* (χ^2=1212, p<0.001). Of the 127 populations containing the F200Y resistant allele, the genotype frequency of 66 populations were significantly different from that which would be expected following the Hardy-Weinberg equilibrium.

2.5 Discussion

The current study identified two SNP mutations in UK *N. battus* populations, previously associated with benzimidazole resistance in other GIN species. F167Y; identified for the first time in this species, was detected at a low frequency in a small number of populations. F200Y was found to be the predominant mutation, as in several other ovine GIN species. This SNP was identified throughout the UK, in one in four of the populations tested, albeit at a low resistant allele frequency in most. North West England was identified as a focal region of resistance, with significantly higher F200Y allele frequencies compared to other regions tested.

The use of molecular genotyping methods to test anthelmintic efficacy provides an opportunity for rapid, accurate results to be generated from a single sample without the need to treat animals and collect post-treatment samples. Analysis of anthelmintic efficacy from a single sample is of particular benefit for *N. battus* as faecal egg count reduction, the current gold standard, can provide spurious results for this species due to the rapid reduction

in adult worm burden caused by immune-mediated hypersensitivity reactions during acute infection. However, the correlation between β-tubulin SNP frequency and anthelmintic efficacy has not been reliably characterised for *N. battus,* or any other nematode species. It is therefore difficult to interpret resistant allele frequency results to determine whether or not a treatment would likely be successful in reducing the parasite burden in treated animals and by how much. Previous studies have suggested that phenotypic correlates of resistance such as continued egg output from infected animals post-treatment or parasite eggs able to hatch when exposed to the therapeutic dose of thiabendazole during *in vitro* egg hatch assays may become evident from resistant allele frequencies of 15-30% (Santos et al., 2017; von Samson-Himmelstjerna et al., 2009). However, the correlation between SNP frequency and anthelmintic efficacy is difficult to estimate given the interplay between multiple SNPs (F167Y, E198A and F200Y), the suggestion that each may have a different phenotypic impact (Kotze et al., 2012) and additional factors such as loss of the β-tubulin isotype 2 gene (Kwa et al., 1993). There is also a likelihood that phenotypic resistance may vary between isolates of the same nematode species (von Samson-Himmelstjerna et al., 2009). Knowledge of the prevalence of SNP mutations associated with BZ-resistance provides a useful benchmark from which to chart the rate of development and spread of resistance.

Given the extent of the current data set, North West England was the only focal region of F200Y identified. Due to time and labour constraints, it was not possible to conduct intensive sampling in all suspected 'focal regions' highlighted by the current study as the aim was to create a picture of the prevalence throughout the UK as a whole. Further sampling in the region surrounding populations with high F200Y frequency such as North East Scotland and South West England may uncover additional focal regions. Within the identified focal region, there were numerous populations in which no resistant alleles were found, suggesting that the drivers influencing the development of BZ-resistance are likely operating at farm-level. Genotyping results from the subset of farms within the focal region which were repeatedly

sampled between 2011 and 2016 showed relatively constant resistant allele frequencies over time. Variation was consistent with that observed in previous studies of BZ-resistant populations over multiple samplings (Scott et al., 1989) and was likely due to the number of samples analysed from each snap shot of the population.

Compared to other GIN species, including other *Nematodirus* species (Oliver et al., 2016a), the emergence of anthelmintic resistance has been slower in *N. battus*, possibly resulting from differences in life history traits between the species. As the first BZ-resistant isolate of *N. battus* was reported around the same time as increasing reports of autumn infections and extended transmission periods in this species, the emergence of BZ-resistance in *N. battus* may be associated with changing epidemiology.

As BZ-resistance in *N. battus* appears to be at an early stage, this species may provide a unique opportunity for in depth study of mechanisms involved in the emergence and dissemination of anthelmintic resistance in gastrointestinal nematodes. As outlined within the general introduction (Chapter 1, section 1.7.5), several potential methods have been described for the development and dissemination of SNPs associated with anthelmintic resistance. Briefly, the mutation could have arisen within a single isolate and disseminated out or it may have multiple origins, resulting from either selection of a pre-existing mutation or from multiple spontaneous mutations. Analysis of *H. contortus* and *T. circumcincta* isolates from the UK and worldwide have previously concluded that F200Y likely arose from multiple mutations in different locations (Chaudhry et al., 2015b; Redman et al., 2015). F167Y and E198A are believed to be rare mutations disseminated from a limited number of sources (Chaudhry et al., 2015a; Redman et al., 2015). The origin(s) of the F200Y and F167Y SNPs cannot be determined from prevalence data however, the detection of F167Y in a small number of geographically diverse locations, both with and independent of F200Y, may

indicate multiple origins. Although this contradicts previous findings in other trichostrongylid species, given the evidence for independent occurrence of BZ-resistance associated SNPs (Chaudhry et al., 2015b; Redman et al., 2015; Silvestre et al., 2009; Skuce et al., 2010) and the suggestion that different SNPs have varying contributions towards phenotypic anthelmintic efficacy (Kotze et al., 2012; Silvestre and Cabaret, 2002), it is perhaps not surprising that F167Y was identified independently of F200Y in the current study. The high prevalence and low frequency of F200Y observed in *N. battus* may also suggest multiple origins with local dissemination from each, similar to previous findings in *T. circumcincta* (Redman et al., 2015). However, the focal region of high resistant allele frequency surrounding the initial BZ-resistant isolate is indicative of local spread from a single source. In which case, the national distribution of F200Y may be due to animal trade. Future study of the involvement of animal trade and movements in dissemination of anthelmintic resistance would be interesting and could be achieved by sampling at livestock markets and agricultural shows. The local spread of resistant alleles between farms within a focal region may also be linked with wildlife and weather events. Wildlife are frequently observed grazing in livestock fields and have been shown to carry GIN typically associated with sheep and cattle, including anthelmintic resistant isolates (Chintoan-Uta et al., 2014; Pato et al., 2013). Patent, viable *N. battus* infections have been demonstrated in wild deer and rabbits (Boag, 1972; Dunn, 1965). It is therefore reasonable to assume that BZ-resistant *N. battus* could be spread by wild deer in the UK given a high resistant allele frequency. A review of historic complaints to the Agricultural Development and Advisory Service regarding damage to agricultural land or crops by wild deer showed that red deer were most commonly associated with pasture and forage crop damage. Other species such as fallow and roe deer were linked to loss of cereals, arable crops and orchard damage (Putman and Moore, 1998). A recent study of the geographic distribution of red deer in the UK illustrated that the North West of England, the focal region of F200Y, has been inhabited by wild red deer consistently since

the study began in 2007 (British Deer Society, 2016). Future research exploring the distribution of *N. battus* within the wild deer population could quantify the biosecurity risk posed by deer to UK sheep enterprises, with a view to developing simple control strategies such as fencing to minimise contact between livestock and wildlife. However, given the clash of timing of *N. battus* peak hatching and typical deer fawning in spring, sample collection may be difficult. Examination of the relatedness between *N. battus* isolated from wild deer and those from nearby farms would provide a valuable insight into parasite gene flow in local regions. Despite the ability of wildlife species to carry resistant GIN and deposit eggs on different farms, the nematode burden and number of parasite eggs deposited on livestock pastures has been estimated to be low (Bartley 2018, *personal communication*.). It is likely then that major parasite movement will be associated with livestock trade, placing greater importance on effective quarantine.

The F200Y locus was found to be under active selection in a number of populations. Therefore, despite few clinical cases of BZ-resistance causing noticeable treatment failure on farm at present, BZ-resistance has the potential to increase in future given the appropriate selection pressure. Monitoring anthelmintic efficacy on farm will therefore be important to ensure effective treatment and reduce morbidity and mortality in lambs. The pyrosequencing method discussed in this chapter provides accurate results and could be utilised as a diagnostic at centralised testing laboratories however, analysis of individual eggs and larvae was time consuming and would require trained technicians. Development of pen-side rapid tests to detect and quantify the SNPs associated with BZ-resistance would be beneficial in this situation, as the test could be performed by a vet on farm, allowing for point-of-care decision making whilst animals are penned.

2.6 Conclusions

The current study identified both the F167Y and F200Y β-tubulin isotype 1 SNPs in UK *N. battus* populations; previously associated with BZ-resistance in other GIN species. F167Y was found to be at a low allele frequency in a small number of populations. F200Y was also identified at a low frequency overall but was present in around a quarter of the populations tested with focal regions of high resistant allele frequency. The presence of F200Y in a large proportion of the populations tested and the finding that this locus is under active selection indicates the potential for this mutation to increase in frequency in the future. The results discussed within this chapter provide a benchmark of resistance in this species, allowing for the progression of BZ-resistance to be followed. As resistance appears to be at an early stage in this species, *N. battus* provides a unique opportunity to study the emergence and spread of resistance to identify management factors capable of slowing the advancement of resistance. Greater understanding of the drivers of resistance may influence management, potentially prolonging the effective lifespan of BZ compounds for use against *N. battus* which would in-turn protect the longevity of the other anthelmintic classes against other GIN species as they would not be relied upon early in the season.

3 Development and evaluation of a loop-mediated isothermal amplification assay for the sensitive detection and quantification of single nucleotide polymorphisms associated with benzimidazole resistance in *Nematodirus battus*

3.1 Abstract

Loop-mediated isothermal amplification (LAMP) has been used in veterinary and medical fields to develop rapid diagnostics and point-of-care (POC) tests. The diagnosis of anthelmintic resistance is ideally suited to POC testing as treatment decisions could be made whilst animals were gathered, minimising time and labour costs associated with re-gathering animals. In the current proof-of-concept study, a LAMP assay was designed and evaluated for the sensitive detection of the single nucleotide polymorphism (SNP) F200Y of the β-tubulin isotype 1 gene which has been associated with benzimidazole-resistance in multiple nematode species and shown to confer resistance in *N. battus* (chapter 2). The prototype assay was designed to selectively amplify only individuals with the resistant allele. By placing the Backward Inner Primer (BIP) binding site over the SNP site, the mismatch of primer and template sequence with susceptible alleles prevented amplification. Six different primer sets (BIP1 – BIP6) were evaluated during the study, each with the SNP site positioned at a different point of the 3' end of the BIP primer. One set (BIP4) was selected for further optimisation and testing based on the specificity and repeatability of results during initial reactions. Reaction temperature, inner/outer primer ratio and the concentrations of dNTPs, $MgSO_4$ and primers were optimised using plasmid DNA standards to maximise the specificity of the reaction. The optimised prototype assay, using gDNA extracted from individual adult *N. battus,* successfully amplified only samples containing the resistant allele when tested. Tests using plasmid DNA consistently amplified the resistant allele ten minutes quicker than

the susceptible allele, allowing for distinction between resistant and susceptible individuals. The prototype assay was also found to be semi-quantitative, amplifying samples in the order of resistant allele frequency however, separation between resistant allele frequencies was minimal. Further evaluation of the prototype assay using field samples produced inconsistent results.

3.2 Introduction

Anthelmintic resistance is an increasing problem, threatening the sustainability of livestock farming and the need for rapid, robust diagnostics is increasing at the same rate. The genetic basis of benzimidazole resistance has been studied in detail with several single nucleotide polymorphisms (SNPs) within the β-tubulin isotype 1 gene being identified (Ghisi et al., 2007; Kwa et al., 1994; Silvestre and Cabaret, 2002) which appear to be conserved between nematode species (Ramunke et al., 2016; Silvestre and Humbert, 2002) and across taxa (Jung et al., 1992; Koenraadt et al., 1992). Characterisation of the initial BZ-resistant *N. battus* population identified that the F200Y SNP mutation, which codes an amino acid change from phenylalanine to tyrosine (TAC-TTC), also conferred resistance to fenbendazole in this species (Morrison et al., 2014). Given knowledge of the genetic basis of BZ-resistance, rapid, point-of-care (POC) diagnostic tests could provide valuable tools, allowing for rapid resistance profiling on farm to inform treatment decisions.

The current practical 'gold-standard' method for estimating anthelmintic efficacy in the field is the faecal egg count reduction test (FECRT) however, this requires treatment of animals and multiple samples collected 7-14 days apart (Coles et al., 1992). For testing *Nematodirus battus*, this poses a particular problem. As mass shedding of adult worms, caused by a hypersensitivity reaction, is a common feature of its immune-epidemiology, after an initial peak, FECs tend to drop very rapidly in untreated animals as well as treated animals. This

renders it hard to deduct the wormer-induced reduction in worm burden and egg counts. The development of a reliable molecular diagnostic test to detect and quantify SNP mutations, from a single pooled sample, could have multiple uses. The test may potentially provide an estimation of how effective anthelmintic treatment is likely to be prior to administration or provide information as to whether an apparent treatment failure occurred as a result of resistance or due to another factor, such as mis-dosing or reinfection. The presence of F200Y has been found to correlate well with benzimidazole efficacy *in vivo* (Cudekova et al., 2010). To date, the detection of SNPs has largely been achieved by two-step process: amplification of the target region by PCR followed by detection of the SNP of interest within the amplicons (section 1.7.7.2.2). Two-step methods such as pyrosequencing provide reliable results but are costly and time-consuming to perform. LAMP offers a rapid, low cost, single-step alternative (section 1.7.7.2.2.3) and has previously been applied to the detection of SNPs (Duan et al., 2014; Yongkiettrakul et al., 2017). Briefly, this amplification technique utilises a set of 4-6 primers which sequentially bind the template DNA and amplify the target by strand displacement to produce large, stem-loop structured amplicons (Nagamine et al., 2002; Notomi et al., 2000). LAMP uses *Bst. polymerase* which rapidly amplifies DNA under isothermal conditions which, together with the development of lyophilised reagents, makes this technology suitable for use in resource limited situations and has led to the translation of this technology into POC devices (Hsieh et al., 2012; Waters et al., 2014; Xu et al., 2016; Yongkiettrakul et al., 2017; Zanoli and Spoto, 2013). Reaction reagents are lyophilised on filter paper, addition of the sample re-hydrates the reagents and initiates the reaction, results can then be visualised by a colour change (Yongkiettrakul et al., 2017). Given the increased accessibility of 3D printing, advances have also been made into the development of microfluidic devices and multi-parallel systems which allow several LAMP assays to proceed simultaneously from a single template sample e.g. Figure 3.1 (Fang et al., 2011; Hsieh et al., 2012). LAMP would therefore be ideal for use on-farm or in

veterinary surgeries, using a crude in-field DNA extraction from faeces which could be directly added to a lateral flow device containing all required LAMP reagents lyophilised within. POC tests would be of specific benefit in livestock farming, particularly in situations where animals are rarely gathered, e.g. hill farms, providing rapid results at, or near, the point of care to assist with on-farm decision making.

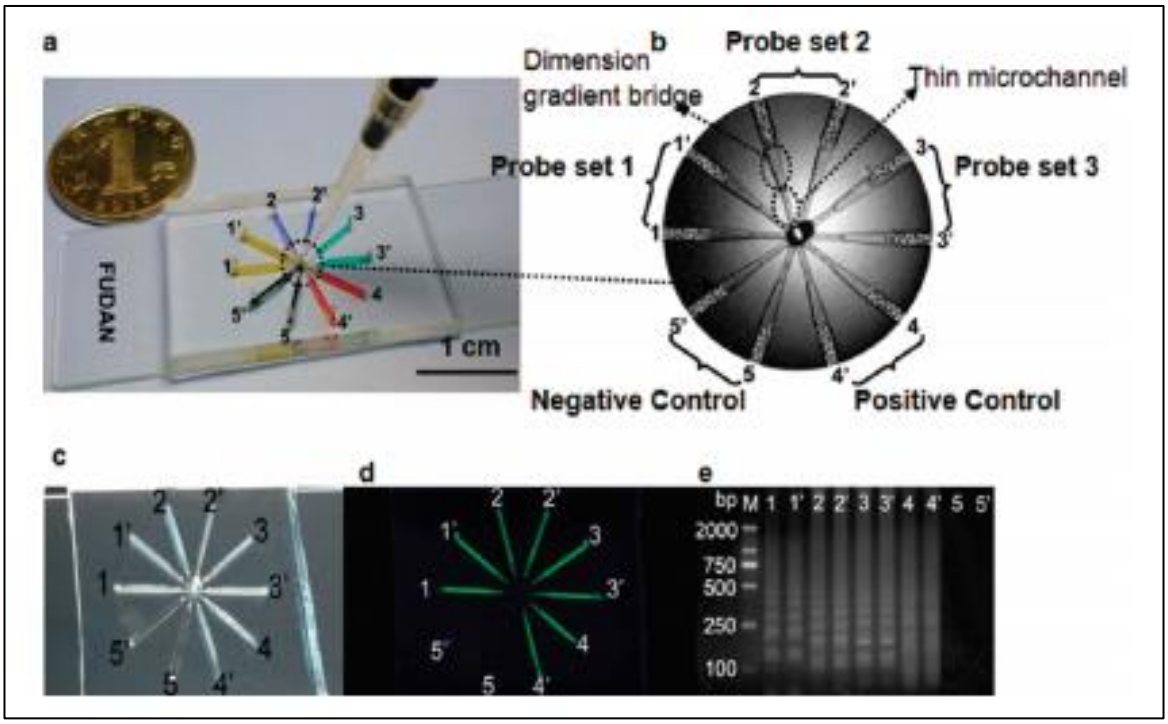


Figure 3.1. Application of loop-mediated isothermal amplification in a point-of-care device.

Figure shows a microfluidic chip design for multiplex LAMP. The chambers each contain lyopholised reagents with different probes for detection of different targets, all fed from a single sample cell in the centre . Reprinted with permission from (Fang et al., 2011). Copyright 2018 American Chemical Society.

LAMP assays have been developed for the detection of a wide variety of human, veterinary and plant pathogens in recent years. Medical diagnostic tests have been developed for several bacterial pathogens and viruses such as *Escherichia coli* and *Ebola* (Dukes et al., 2006; Hill et al., 2008; Kurosaki et al., 2007; Oloniniyi et al., 2017; Poon et al., 2004; Xu et al., 2016). When it comes to parasite diagnostics, LAMP research has focused largely on neglected

tropical diseases such African sleeping sickness (Njiru et al., 2008), visceral leishmaniasis (Khan et al., 2012) and the quantification of Loa Loa infections (Drame et al., 2014). The Loa Loa LAMP assay was designed as a POC test for use during mass ivermectin treatment campaigns, aimed at controlling onchoceriasis and lymphatic filariasis. The test was designed to identify individuals co-infected with Loa Loa for whom the ivermectin treatment could be potentially life threatening.

Veterinary uses include diagnostics for a range of bacterial (Diribe et al., 2014; Karthik et al., 2014), viral (Fan et al., 2012; Le Roux et al., 2009; Mulholland et al., 2014; Parida et al., 2004) and parasitic (Iseki et al., 2007; Melville et al., 2014) pathogens of livestock and companion animals. A few assays have been published for the detection and confirmation of veterinary helminth species, largely restricted to trematode parasites; *Fasciola hepatica* and *Opisthorchis viverrini* (Ai et al., 2010; Arimatsu et al., 2012) and detection of the gastrointestinal nematode *Haemonchus contortus* from parasite eggs in faecal samples (Melville et al., 2014). The development of a rapid, pen-side test capable of detecting and potentially quantifying the SNP mutations associated with BZ-resistance could pave the way for future development of anthelmintic resistance detection panels which could revolutionise helminth control in livestock. The cost of production loss resulting from helminth disease in the UK is difficult to quantify. Estimated at £4.40 per lamb in 2005, based on lamb finishing time assuming a 10% reduction in daily weight gain from parasitic gastroenteritis (Nieuwhof and Bishop, 2005), and will likely have increased in recent years in line with increasing anthelmintic resistance (Thomas et al., 2015). In the case of *N. battus*, infected lambs may shed large numbers of eggs prior to diagnosis and treatment, particularly if testing for BZ-resistance post treatment. However, reliable diagnosis of anthelmintic resistance would be beneficial in the design of effective control strategies for future years, to reduce pasture contamination and disease severity which translates into increased

productivity and economic benefits for future years, improving the sustainability of livestock farming.

The current study employed selective amplification in which the SNP of interest was placed within the forward and backward inner primer (FIP/BIP) binding sites, preventing amplification of the opposite allele due to primer/sequence mismatch. The stringency of primer binding and placement of the SNP within the primer binding site are key factors in the success of these assays. Alternative methods include probes which bind the SNP site to block amplification (Minnucci et al., 2012) or detection of the SNP post-amplification (Gadkar et al., 2018; Mori et al., 2006; Veigas et al., 2013).

The aim of the current study was to develop, optimise and validate an allele-specific LAMP assay capable of detecting the F200Y mutation. The initial development was conducted on plasmid and gDNA generated from adult N. battus worms/larvae with a view to developing an assay that worked on pooled *N. battus* eggs from field derived faecal samples.

3.3 Methods

3.3.1 Preparation of gDNA standards

Genomic DNA (gDNA) standards had been previously prepared from single adult male *N. battus* recovered post-mortem from lambs in the controlled efficacy test described by Morrison *et al.* (2014). Briefly, adult *N. battus* worms were picked from EtOH-fixed small intestine contents, sexed and species identified microscopically. Genomic DNA was extracted using DNeasy blood and tissue kit (Qiagen, Germany) from 20 individual adult males from the resistant isolate control (un-treated), 20 resistant isolate post-fenbendazole administration (at 5mg/kg bodyweight) and 20 from a BZ-susceptible isolate, resulting in 60 individual gDNA samples. Following DNA extraction, samples were genotyped for the F200Y

SNP to confirm resistant or susceptible alleles using triplicate pyrosequencing assays as described in the paper by Morrison *et al.* (2014). The genotyped DNA samples were then used as gDNA controls in the current assay development study.

3.3.2 Preparation of plasmid DNA standards

The β-tubulin DNA fraction of interest, containing the three potential SNP sites at codons 167, 198 and 200 was amplified using F3/B3 primers (Table 3.2) for insertion into plasmids. One BZ-susceptible (TAC/TAC at codon 200), one heterozygote (TAC/TTC) and two BZ-resistant (TTC/TTC) gDNA control samples were used. Using Novataq hotstart PCR mix (Merck, USA), PCR reactions were set up in 50µl volumes containing 25µl reaction buffer, 1.5µM $MgCl_2$, 0.2µM forward and reverse primers and 2µl of template DNA. Reactions were incubated at 95°C for 10 minutes followed by 35 cycles of 94°C for 30 seconds, 51°C for 30 seconds and 72°C for 30 seconds, with a final extension phase at 72°C for 10 minutes. PCR products were visualised on a 2% agarose gel using gel red (Bioline, UK) and product bands were excised from the gel. PCR products were cleaned using the Wizard SV gel and PCR clean-up system (Promega, USA) following the manufacturers protocol.

Ligation reactions were carried out using the pGEM-T easy vector system (Promega, USA) and One shot Mach1-T1R chemically competent *E. coli* cells (Invitrogen, USA) were transformed with the plasmids following the manufacturer's protocol. Transformed cells (30µl) were spread onto pre-warmed selective plates containing 10µg/µl ampicillin and 50 mg/ml X-Gal, plates were incubated at 37°C overnight to allow colonies to grow. Eight white colonies were picked per sample and the presence of the plasmid was confirmed by colony PCR. PCR reactions were set up in 10µl volumes containing 5µl reaction buffer (Novataq hotstart mastermix, Merck, USA), 1.5µM $MgCl_2$, 0.2µM forward and reverse primers (F3 and B3, Table 3.2) and 1µl of template; molecular grade water inoculated with the selected colony. Reactions were run using the PCR conditions detailed above and amplification was

visualised on a 2% agarose gel. Following screening, one colony from each sample was re-picked and used to inoculate warm LB-broth containing 100µg/ml ampicillin, cultures were incubated overnight at 37°C with shaking and cultures were cleaned using Wizard Plus SV minipreps DNA purification system (Promega, USA) following the manufacturers protocol. Plasmid DNA was aliquoted and diluted to 1ng/µl for use in detailed optimisation of primer set BIP4.

3.3.3 Preparation of individual and pooled field samples

DNA lysates were prepared as described in chapter 2. Briefly, ethanol fixed eggs and larvae were re-suspended in 1x phosphate buffered solution (PBS) (1:100 v/v) for 30 minutes to rehydrate. 30 individual eggs/L_3 per *N. battus* population were picked (in 1µl PBS) into individual wells of a 96 well plate (Axygen, USA), containing 15µl lysis buffer (50mM KCl, 2.5mM $MgCl_2$, 10mM Tris (pH 8.3) 0.45% Nonidet P-40, 0.45% Tween 20, 0.01% Gelatine) (Kwa et al., 1995). Eggs and L_3 were subjected to eight cycles of 30 seconds freeze in liquid nitrogen followed by 1 minute incubation at 100^{o}C to weaken the egg shells. A further 15 µl of worm lysis buffer containing 0.2 mg/ml proteinase K (Promega, USA) was added to each well of the plate. Plates were incubated at 56^{o}C overnight and the temperature was then increased to 92^{o}C for 10 minutes to deactivate the proteinase K. Crude lysates were then tested individually or 5µl from each well was pooled for use as template in LAMP reactions. A total of 54 individual eggs and 13 L_3 were analysed by LAMP during the current study.

3.3.4 Pooled *N. battus* L_3 extracts

Larvae from a *N. battus* isolate with high F200Y frequency (>98%) and a BZ-susceptible isolate (0% F200Y) were microscopically picked out in the numbers detailed in Table 3.1.

DNA was extracted using DNeasy blood and tissue kit (Qiagen, Germany). Individual L_3 were picked in 0.5µl of 1X PBS into 180µl of ATL buffer provided in the kit, the extraction was carried out following the manufacturers' protocol and DNA was eluted in a 100µl volume.

Table 3.1. Details of pooled L_3 DNA extractions.

Number of resistant and susceptible larvae included in pooled L_3 DNA extractions and the expected resistant allele frequency of the resultant mixture.

Sample	BZ-susceptible L_3	BZ-resistant L_3	Expected resistant allele frequency
1	100	-	0
2	99	1	0.01
3	95	5	0.05
4	90	10	0.10
5	75	25	0.25
6	50	50	0.49
7	25	75	0.74
8	10	90	0.88
9	5	95	0.93
10	1	99	0.97
11	-	100	0.98

3.3.5 Sequencing of the β-tubulin isotype 1 gene in *N. battus*

The required fragment of β-tubulin isotype 1 gene containing the three potential SNP sites at codons 167, 198 and 200 was amplified using degenerate primers previously shown to amplify this fragment in *Trichuris* species from a variety of host species (Hansen et al., 2013). PCR reactions were performed using Platinum taq polymerase (Invitrogen, USA) in a final volume of 25µl containing 12.5µl reaction buffer, 1.5mM MgCl2, 0.2mM dNTPs, 0.4mM beta-

DF2 (AAYTGGGCKAARGGSCACTA) and beta-DR1 (GWGGATCACAAGCWGCCATC) primers, 0.2µl taq polymerase and 1.5µl DNA template (gDNA control samples). Reactions were incubated at 94˚C for 10 minutes followed by 30 cycles of 94˚C for 30s, 55˚C for 30s and 72˚C for 30s with a final extension phase at 72˚C for 10 minutes.

PCR products were visualised on a 2% agarose gel with gel red (Biotium, USA) and PCR product bands were excised. PCR products were purified from the gel fragments using the Wizard SV gel and PCR purification kit (Promega, USA) following the manufacturers' protocol. Purified DNA was quantified using a Nanodrop spectrophotometer. DNA samples were diluted to 5ng/µl and sent to MWG Eurofins (Germany) for sequencing in both the forward and reverse directions. The sequences were aligned and analysed using DNASTAR Lasergene software.

3.3.6 Workflow of design and evaluation

The workflow of the study is outlined in Figure 3.2. Each of the six primer sets were initially tested using reagents reconstituted in CHES CAPSO and Tris buffers (detailed in section 3.4.2). Primer sets BIP1-4 were also tested with and without loop primers to assess the speed of reaction and the impact on specificity (3.4.2). Repeatability tests were then carried out using primer sets BIP1, 3 and 4 (3.4.3). BIP4 primers were selected for further optimisation (3.4.4); $MgSO_4$ concentration, reaction temperature, dNTP concentration and inner/outer primer ratio and concentration were all optimised using plasmid DNA. The optimised prototype assay was then tested using field derived material (3.4.6) and the ability of the test to quantify the resistant allele frequency was evaluated (3.4.5).

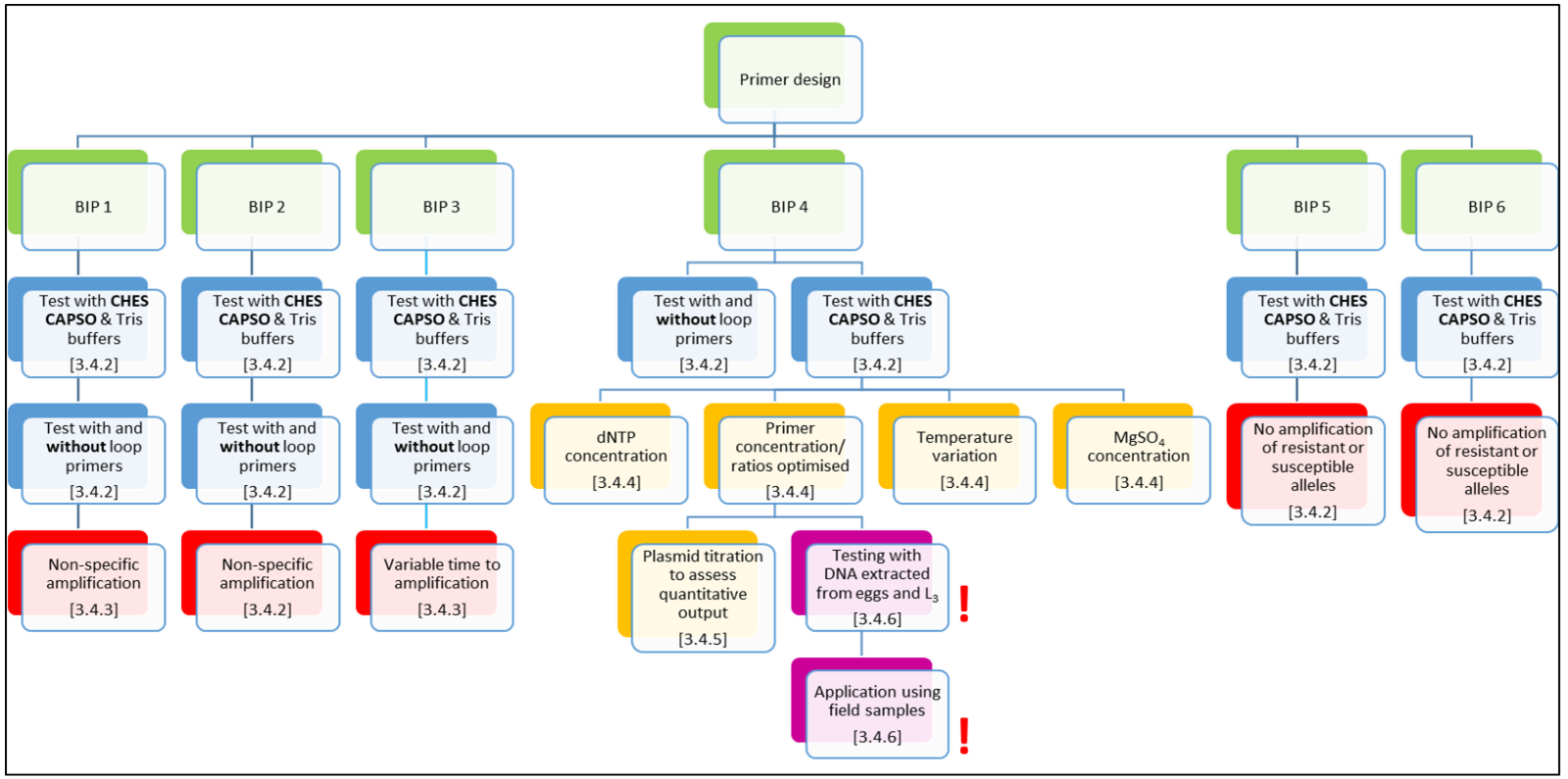


Figure 3.2. Work flow of LAMP assay design and evaluation.

The colour of the boxes indicates the template DNA used during this part of development; blue represents gDNA controls extracted from single adult N. battus of known genotype, yellow is plasmid DNA containing either susceptible or resistant alleles and pink is gDNA extracted from eggs and/or L_3 from field-derived N. battus *populations. Red boxes indicate when development of a primer set was stopped and the red exclamation marks highlight areas where results were inconsistent. The numbers in square brackets indicate the relevant results section.*

3.3.7 Primer design

Primers were designed by Mast Ltd (Liverpool, UK) using the Primer Explorer software package, based on gDNA sequences from BZ-resistant and susceptible *N. battus* individuals, to target the β-tubulin isotype 1 gene. Several different primer designs have been published in the literature for SNP-detection LAMP assays in which the placement of the SNP of interest varied within the B2/F2 region of the BIP/FIP primers (Badolo et al., 2012; Duan et al., 2014; Minnucci et al., 2012; Zhang et al., 2016; Zhang et al., 2015) and for this reason, a total of six primer sets (BIP1 – BIP6) were designed and tested in the present study with varying SNP placement within the BIP primer binding site. Optimised LAMP conditions were previously set out in the literature (Notomi et al., 2000), the primer sets and assay design in the current study were devised to fit the optimised conditions for target size (130-200bp), loop size (40bp or longer) and enzyme choice (*Bst.* polymerase). Notomi *et al.* (2000) suggested that the T_m of the F2 and B2 regions of the FIP and BIP primers should be between 60 and 65˚C to coincide with the optimum working temperature for *Bst.* polymerase and that the T_m of the F3 and B3 primers was lower than that of F2 and B2 to ensure the amplification began from the inner primers (FIP and BIP, of which F2 and B2 are a part) rather than the outer primers (F3 and B3).

The sequence of primers F3, B3, FIP, Loop F and Loop B were the same for primer sets BIP1-5 (Table 3.2). BIP 1-3 were designed with the same B1 region (bold text in Table 3.2) and B2 (regular text) varied in length and SNP position (bold, red text). The B2 region was 20bp long in BIP1 with the SNP on the second last nucleotide, the SNP was in the same position in the BIP2 primer and the B3 region was 21bp long, the B2 region of BIP3 was 19bp long with the SNP positioned three nucleotides from the end of the primer (Table 3.2). BIP4 and BIP5 were designed following methods described by a previous study (Badolo et al., 2012) in which the SNP was positioned on the final nucleotide of the primer (BIP4) and included an additional

mismatch nucleotide on the second last base (BIP5). BIP6 was designed following the methods described in additional studies (Yongkiettrakul et al., 2017; Zhang et al., 2015) where the SNP of interest is placed within the binding site of both inner primers (FIP and BIP) (Table 3.2). Primer set BIP6 was designed without using the primer explorer software as the program did not allow for overlapping primer binding sites (Notomi et al., 2000).

Table 3.2. Primer sequences designed during the LAMP development study.

The outer primers (F3/B3), inner primer FIP and loop primers F & B were common to each primer set (blue shaded). The F1 and B1 regions of the FIP and BIP primers respectively, are highlighted in bold text. The single nucleotide polymorphism is highlighted in bold red text in each BIP primer, the lower-case letter in BIP mismatch is an additional mismatch base included to increase binding specificity.

Primer	Sequence
F3	AGG ATT ATG GCA TCA TTC TCT G
B3	CAT AGG TGG GAT TTG TGA GTT
FIP	**ACC TTG AAA AGT TGC GAT GTT AAC G**GG TAG GTG TGG CCT ATC A
Loop F	GTT CGG CAT TTA ACG AAG AGC
Loop B	CTC TCT GTT CAT CAG TTG GTA GAA
BIP1	**CGT TGT TGA GCC GTA CAA TGC** GCT TCG TTG TCA ATG CAG **T**A
BIP2	**CGT TGT TGA GCC GTA CAA TGC** AGC TTC GTT GTC AAT GCA G**T**A
BIP3	**CGT TGT TGA GCC GTA CAA TGC** CTT CGT TGT CAA TGC AG**T** AA
BIP4	**CGT TGT TGA GCC GTA CAA TGC** GCT TCG TTG TCA ATG CAG **T**
BIP5	**CGT TGT TGA GCC GTA CAA TGC** GCT TCG TTG TCA ATG CAG g**T**
F3 (BIP6)	CTT TAA CGT TAA CAT CGC AAC
B3 (BIP6)	TGA CAC CCG ACA TTG TAA C
FIP (BIP6)	**TAA GTT TCA TCT GTG TTT TCT CCC** CGT TGT TGA GCC GTA C
BIP6	**ACT GCA TTG ACA CCG AGG C**TA GAT GGT TCA GAT CTC C

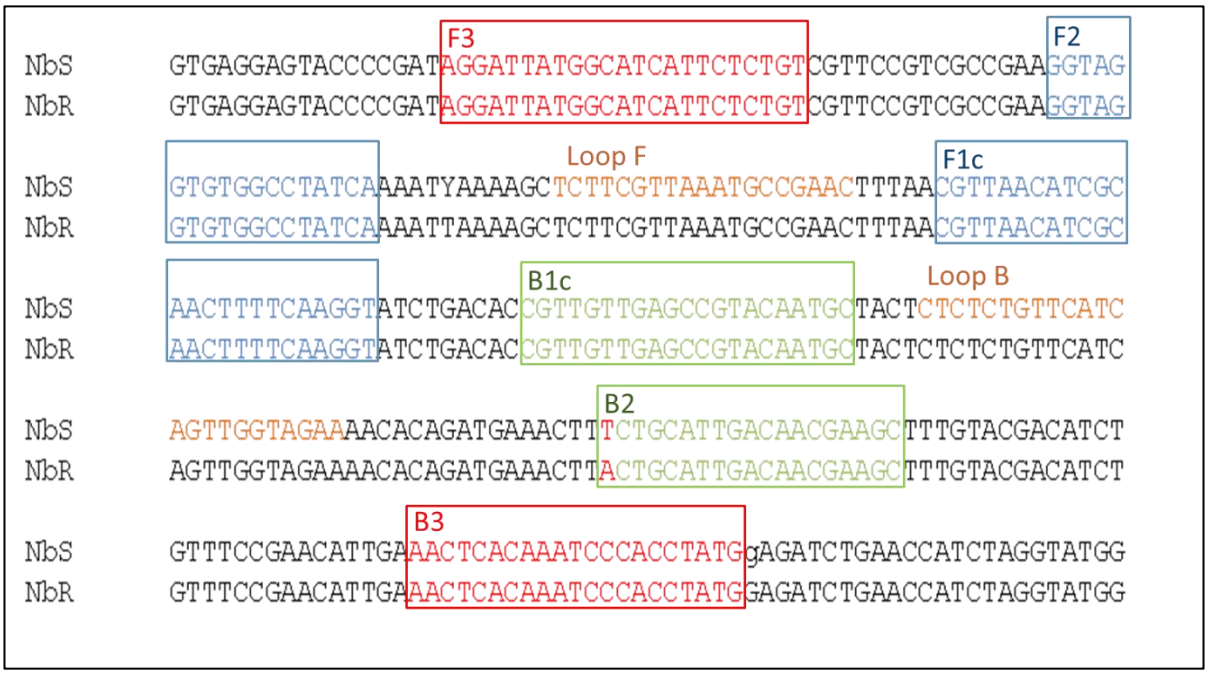


Figure 3.3. Sub-section of the DNA sequence of the N. battus *β-tubulin gene with LAMP primers highlighted.*

Outer primers F3/B3 are highlighted in red, loop primers in orange, forward inner primer (FIP) in blue and reverse inner primer (BIP) in green. The F200Y SNP is highlighted in bold red letters within the B2 region of the BIP primer. FIP and BIP primers each contain two regions; the B2 region which binds first to initiate amplification and the B1c region; this is a sequence complimentary to the DNA sequence. Once the B1 region has been amplified and displaced, the B1c primer region binds back on the growing DNA strand to form the characteristic dumbbell shape required for amplification to continue.

3.3.8 LAMP reaction set up

3.3.8.1 Lyophilised V6.21 pellet reagents

The V 6.21 pellets (Mast Ltd, UK) contained all LAMP reagents including enzyme, nucleotides and detection dye lyophilised into a pellet to be re-constituted in either CHES CAPSO or TRIS buffer. Reactions were run on a real time PCR platform, the dye included in the pellets was an intercalating fluorochrome dye which bound double stranded DNA and produced a light

signal when double stranded DNA molecules were denatured. Results were analysed within the FAM wavelength channel.

V6.21 pellets were reconstituted in 86µl of the chosen buffer (Tris 0.1-1M or CHES CAPSO buffer 0.1-0.25M). Typical LAMP reaction was carried out in a 10µl reaction volume containing 8.6µl buffer reagents reconstituted in 0.25M CHES CAPSO, 0.32µM FIP/BIP primers, 0.08µM F3/B3 primers and 1µl DNA template. The reaction was incubated at 63°C for 55 minutes on a real-time PCR machine, fluorescence was recorded every 33 seconds using the FAM filter unless otherwise stated.

3.3.8.2 Detailed optimisation of primer set BIP4

Detailed optimisation reactions were conducted using *Bst.* 2.0 DNA polymerase (New England Biolabs, USA) to allow for independent variation of each reagent to optimise reaction conditions.

The standard reaction was carried out in 13µl, containing 1.25µl 10X isothermal amplification buffer, 1.2mM dNTPs, 0.32µM FIP/BIP primers, 0.08µM F3/B3 primers, 0.32U *Bst.* 2.0 DNA polymerase, 8mM MgSO4, 0.5µl fluorescent dye (New England Biolabs, USA) and 1µl DNA template. Reactions were prepared on ice and incubated at 63°C for 55 minutes on a real-time PCR machine, fluorescence was recorded every 33 seconds using the FAM filter.

Duplicate resistant and susceptible allele plasmid DNA controls (1ng/µl) were run in each optimisation reaction with one negative control containing molecular grade water. Reagent and reaction condition variations were as follows.

3.3.8.2.1 Magnesium sulphate concentration

Magnesium sulphate ($MgSO_4$); required for optimal activity of the polymerase, also influences the specificity of primer binding similar to PCR (Lorenz, 2012). The isothermal amplification buffer supplied with the *Bst.* 2.0 DNA polymerase (New England Biolabs, USA)

contained 2mM of $MgSO_4$. The manufacturer's protocol recommends 8mM $MgSO_4$ for LAMP reactions, duplicate reactions were run, as detailed in section 3.3.8.2, using 2mM, 4mM, 6mM and 8mM $MgSO_4$.

3.3.8.2.2 Reaction temperature

Repeat reactions were set up as detailed in section 3.3.8.2 for the standard reaction and were incubated at 60°C, 63°C and 66°C all other conditions were kept constant.

3.3.8.2.3 Primer ratio variations

Typical ratio for LAMP was 4:1 inner (FIP/BIP): outer (F3/B3) primers, initial LAMP trials were conducted with 0.32µM FIP/BIP and 0.08µM F3/B3 primers. The prototype assay was tested at primer ratios from 4:1 to 10:1 (Table 3.3).

Table 3.3. Inner (FIP/BIP) and outer (F3/B3) primer concentrations included in primer ratio variation.

Primer ratio	**FIP/BIP concentration**	**F3/B3 concentration**
4:1	0.32µM	0.08µM
6:1	0.48µM	0.08µM
8:1	0.64µM	0.08µM
10:1	0.80µM	0.08µM

3.3.8.2.4 Denaturation step

DNA samples were aliquoted into reaction tubes with or without LAMP primers and incubated at 95°C for 5 minutes then placed on ice for 5 minutes. Tubes were centrifuged to collect the liquid and LAMP reagents were added, the reactions were then incubated as described in section 3.3.8.2.

3.3.8.2.5 Quantification tests

3.3.8.2.5.1 Plasmid DNA

BZ-resistant and susceptible plasmid DNA was titrated from 1ng/µl down to 1pg/µl with molecular grade water. Titrated samples were run using the BIP4 primer set. The lyophilised pellet reagents were re-suspended in 0.25M CHES CAPSO buffer, 0.64µM FIP/BIP, 0.16µM F3/B3 primers and 1µl template were added and the reaction was incubated at 63°C.

3.3.8.2.5.2 Genomic DNA

Control gDNA samples were mixed to produce gDNA samples at approximately 5, 10, 25, 50, 75, 90 and 95% resistant allele frequency. Resistant and susceptible gDNA mixtures and control gDNA samples were tested using the with BIP4 primers at 8:1 (inner: outer) primer ratio at 63˚C.

3.4 Results

3.4.1 β-tubulin isotype 1 gene sequence

Sequencing of the fraction of the β-tubulin gene containing the F167Y, E198A and F200Y SNPs was successful, the alignment of forward and reverse sequences produced a consensus sequence of 801bp (Appendix 1).

3.4.2 Initial testing of primer sets

Each primer set was tested in CHES CAPSO and Tris buffers, with and without loop primers, using gDNA extracted from individual adult *N. battus* worms of known genotype. The results of initial tests are summarised in Table 3.4 and Figure 3.5.

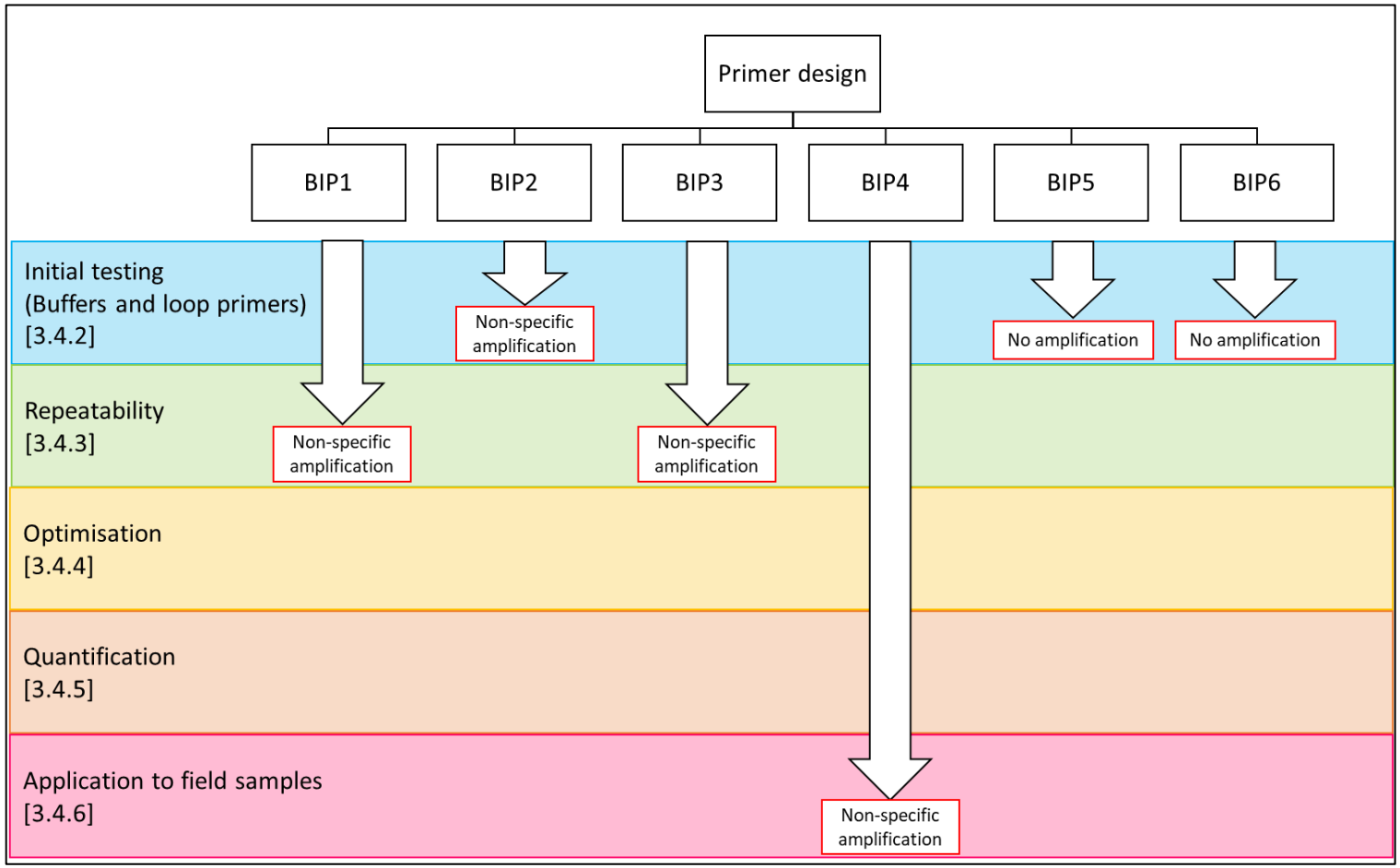


Figure 3.4. Progress chart of LAMP primer sets.

The workflow diagram indicates the stage that each primer set was evaluated to and the respective chapter section relating to each developmental/evaluation stage.

Table 3.4. Results of initial tests using each primer set.

*The table summarises the initial tests conducted for each primer set, detailing time to amplification in minutes, * denotes no amplification within 60 minutes. Each primer set was initially tested in CHES CAPSO and Tris buffers, with and without loop primers.*

Primer set	**Buffer**	**Loop primers**	**Time to amplification (minutes)**	
			Resistant allele	Susceptible allele
BIP 1	CHES CAPSO	Yes	30.3	32.5
		No	32.5	*
	Tris	Yes	*	*
		No	*	*
BIP 2	CHES CAPSO	Yes	25.9	27.0
		No	*	*
	Tris	Yes	42.4	46.2
		No	*	*
BIP 3	CHES CAPSO	Yes	8.3	10
		No	22	*
	Tris	Yes	7.7	9.4
		No	23.1	50.6
BIP4	CHES CAPSO	No	24.8	*
	Tris	No	*	36.9
BIP 5	CHES CAPSO	No	*	*
	Tris	No	*	42.4
BIP 6	CHES CAPSO	No	*	*
	Tris	No	*	*

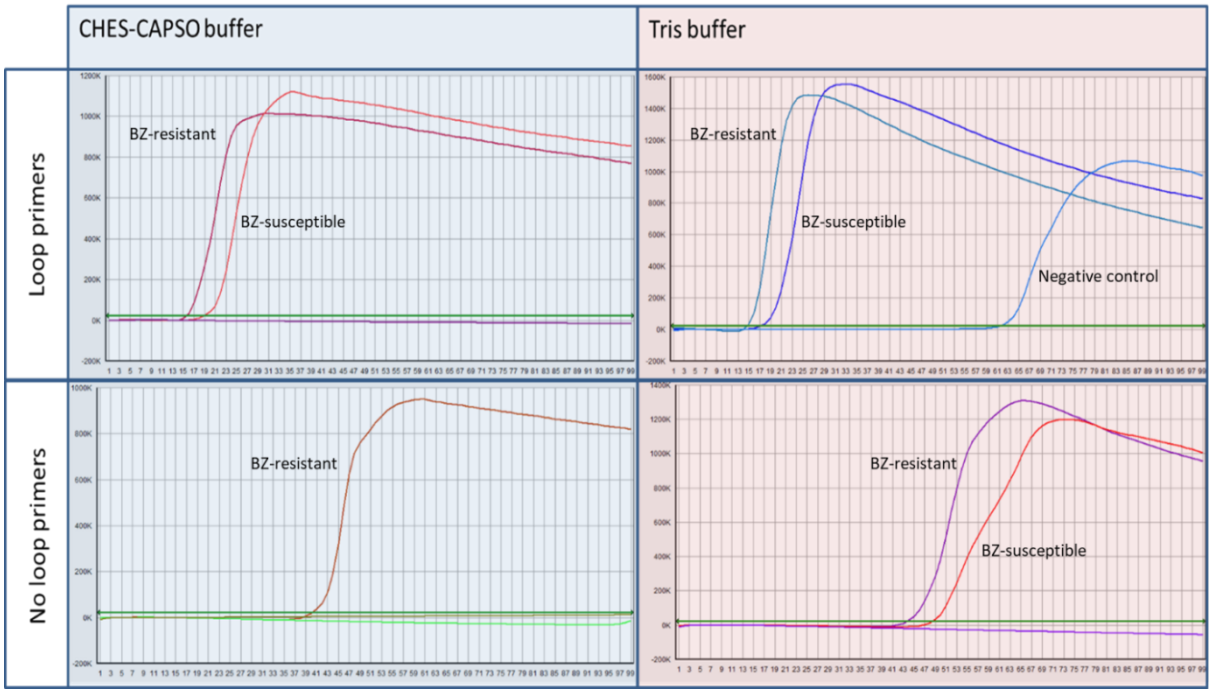


Figure 3.5. Amplification plots of initial tests conducted using the BIP3 primer set.

Results from the amplification of gDNA controls using BIP3 primers, with and without loop primers in CHES-CAPSO and Tris buffers.

The specificity of amplification was found to be greater using the CHES CAPSO buffer compared to Tris with all primer sets. The addition of loop primers increased the speed of the reactions however, specificity was reduced (Figure 3.5). BIP1 primers specifically amplified the resistant alleles without loop primers in a CHES CAPSO background, however, the addition of loop primers resulted in non-specific amplification of the F200Y resistant and susceptible genotypes at approximately the same time. Primer sets BIP3 and BIP4 also showed specific amplification of the resistant allele with the CHES-CAPSO buffer without loop primers. Testing of the same gDNA samples using a Tris buffer and/or adding loop primers induced non-specific amplification of susceptible genotype. No amplification of resistant or susceptible alleles was observed using the BIP2, BIP5 or BIP6 primer sets during initial test reactions and evaluation of these primer sets was stopped at this point.

3.4.3 Repeatability testing

Primer sets BIP1, BIP3 and BIP4 were repeatedly tested to evaluate the robustness of the results observed during initial test reactions using gDNA samples extracted from individual adult worms. Primer set BIP1 was tested in three reactions including 8 DNA samples, BIP3 and BIP4 were testing over 15 reactions including 19 samples and 7 reactions including 30 samples respectively. Repeatability tests were all run using the CHES CAPSO buffer excluding loop primers as these conditions were previously found to provide the most specific amplification for each of the primer sets.

The BIP1 primer set was tested using a further six gDNA samples extracted from individual adult *N. battus*; three resistant and three susceptible genotypes. Repeated trials identified non-specific amplification of the susceptible allele in two of the three susceptible genotype samples tested and failure to amplify one of the resistant genotype samples (Figure 3.6).

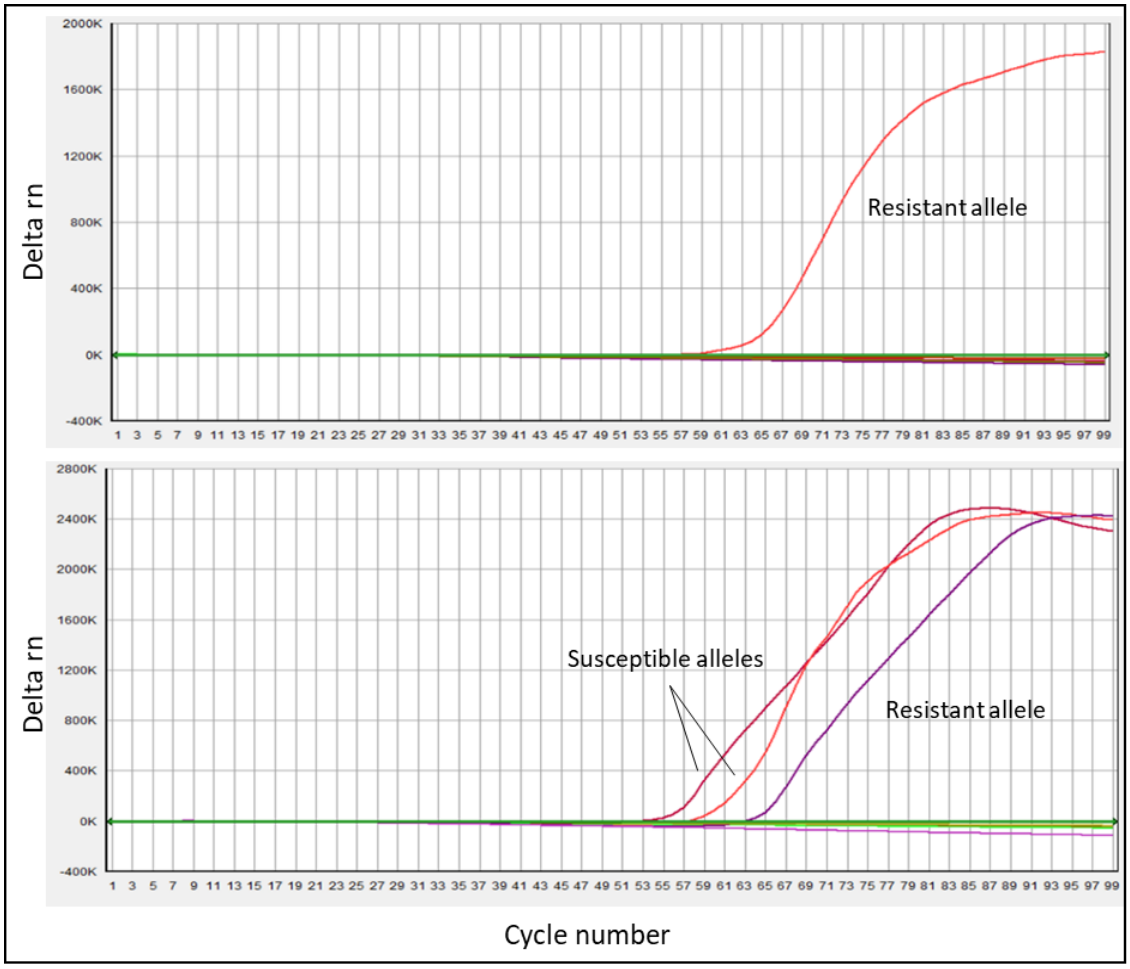


Figure 3.6. Repeatability tests using BIP1 primer set.

Amplification of resistant and susceptible alleles, tested using gDNA standards. Reagents were reconstituted in a CHES CAPSO buffer without loop primers.

Figure 3.7 shows the results of three replicate reactions with primer set BIP3, amplifying the same gDNA samples in each reaction; three susceptible and one resistant genotype sample. The susceptible genotype was amplified in two of the three reactions. The resistant allele consistently amplified quicker than the susceptible allele control however, the timing of amplification varied between tests by approximately 4 minutes. The susceptible allele amplified approximately 10 minutes later than the resistant genotype in trial 1 however, separation was reduced to around 3 minutes in trial 2.

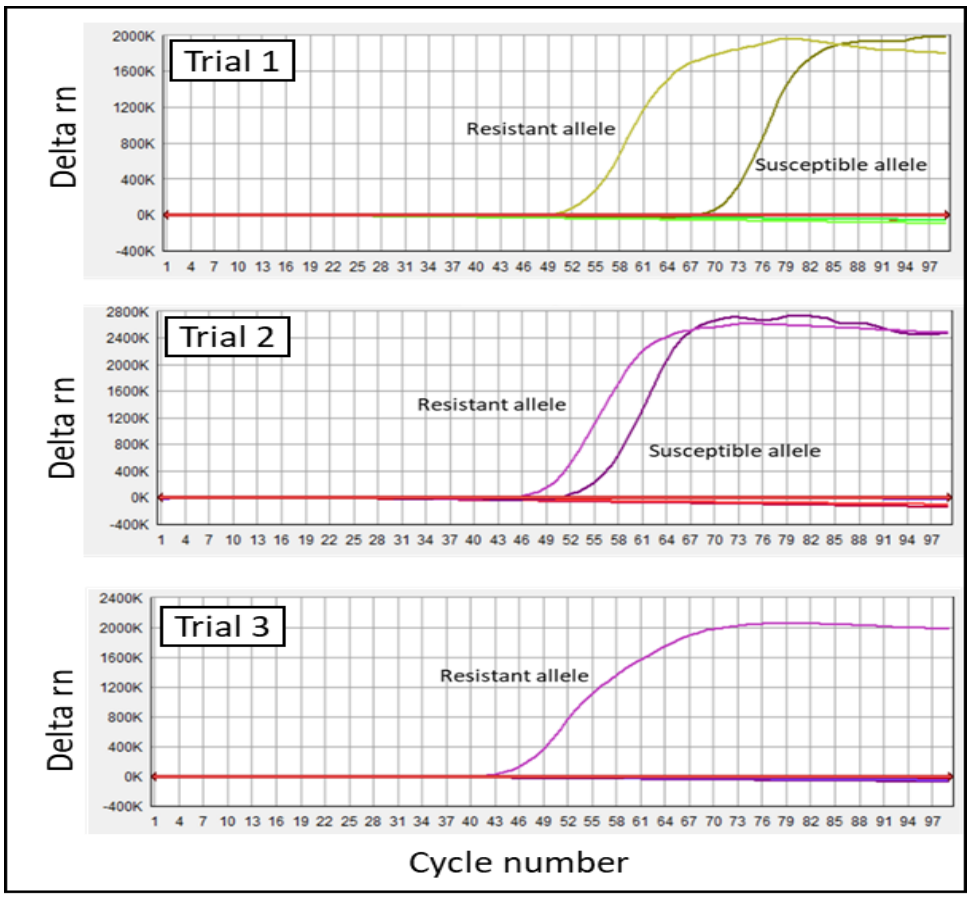


Figure 3.7. Repeated LAMP reactions using BIP3 primer set.

Repeated tests using gDNA standards showing inconsistent results. DNA samples and reaction conditions were consistent between reactions; CHES CAPSO buffer without loop primers.

Four replicate reactions were carried out using the BIP4 primer set, template gDNA samples (one resistant and five susceptible genotypes) were kept constant between tests. In all four reactions, the resistant allele consistently amplified at 45 cycles (approximately 25 minutes) with no false amplification of the susceptible alleles.

No further optimisation was carried out for primer sets BIP1 and BIP3 due inconsistent results and non-specific amplification of susceptible genotypes at a similar time as resistant alleles. The BIP4 primer set was selected for further optimisation of the prototype assay.

3.4.4 Optimisation

Initial testing of the prototype BIP4 assay with plasmid DNA samples showed amplification of both the F200Y resistant and susceptible genotypes, however the amplification time was consistent between replicates and the separation between resistant and susceptible allele amplification was greater than 10 minutes (Figure 3.8).

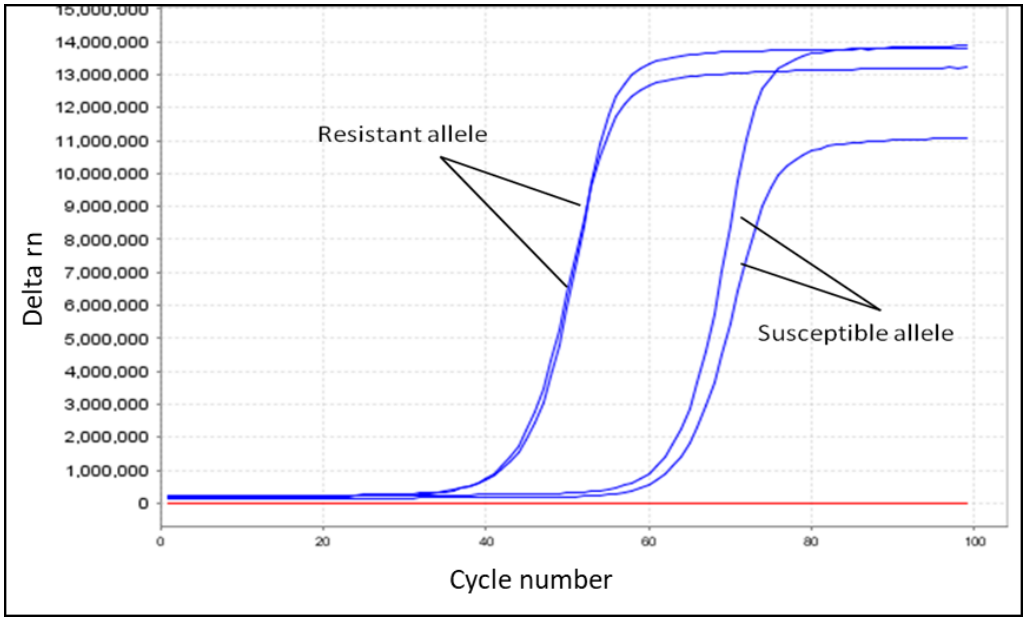


Figure 3.8. Prototype F200Y LAMP assay amplifying plasmid DNA.

Real time fluorescence amplification trace of prototype F200Y BZ-resistant N. battus LAMP assay amplifying plasmid DNA produced from resistant and susceptible isolates, both at 1ng/µl.

Table 3.5 summarises the results of optimisation tests on the reaction conditions of the BIP 4 prototype assay, varying primer ratio and concentration, reaction temperature and the concentration of dNTPs and $MgSO_4$.

Table 3.5. Summary of LAMP optimisation tests.

Mean time to amplification of the resistant and susceptible alleles from plasmid DNA and the difference in amplification time between the alleles using BIP4 primers at varying inner (FIP/BIP): outer (F3/B3) primer ratios, reaction temperature, dNTP concentration and MgSO4 concentration.

Factor altered	Measurement of factor	Number of replicates	Time to amplification (mins (range))		Difference in amplification time between resistant and susceptible alleles (mins)
			Resistant	Susceptible	
Primer ratio (inner: outer)	4:1	2	22 (22-22)	29 (29-29)	7
	6:1	2	15 (15-15)	19 (19-19)	4
	8:1	2	13 (13-13)	17 (16-17)	4
	10:1	2	10 (9-10)	14 (14-14)	4
Primer concentration at 4:1 ratio (inner/outer)	0.64 / 0.16 µM	2	22 (20-23)	31(23-38)	9
	0.48 / 0.12 µM	2	29 (20-37)	44 (23-64)	15
	0.32 / 0.08 µM	2	25 (24-25)	41 (33-48)	16
Reaction temperature	60	2	18 (17-18)	26 (26-26)	8
	63	2	23 (23-23)	38 (37-38)	15
	66	2	23 (20-25)	32 (32-32)	8
dNTP concentration	0.8mM	2	32 (30-34)	44 (43-45)	12
	1.2mM	2	23 (23-23)	38 (37-38)	15
MgSO4 concentration	2mM	2	21 (20-22)	32 (31-32)	11
	4mM	2	28 (27-28)	39 (38-39)	11
	6mM	2	37 (36-37)	44 (43-46)	7
	8mM	2	28 (27-30)	33 (32-34)	5

Increasing the concentration of inner primers (FIP/BIP) compared to outer primers (F3/B3) was expected to increase the stringency of primer binding however, the difference in amplification time between resistant and susceptible alleles was found to be greatest at 4:1 primer ratio (Table 3.5). Time to amplification of the resistant allele was also found to vary with the ratio of inner: outer primers; faster amplification of the resistant allele was observed with increasing inner primer concentration; 10 minutes using 10:1 ratio, increased to 22 minutes with 4:1 ratio.

Primer concentration three (0.32/0.08 µM inner/outer primers) provided the greatest separation between amplification of resistant and susceptible alleles (average 15.9 minutes). Unlike primer concentrations one and two (0.64/0.16 and 0.48/0.12 µM inner/outer primers respectively), the time to amplification of both alleles was consistent and did not overlap between repeated reactions.

The speed of the reaction was found to be inversely correlated with reaction temperature; resistant allele amplified after 18 minutes at 60˚C and 24 minutes when incubated at 66˚C. The greatest difference in amplification time between resistant and susceptible alleles was observed at 63˚C; 15 minutes between resistant and susceptible allele amplification compared to 8 minutes at 60 and 66˚C.

Reducing the dNTP concentration slowed the reaction; the F200Y resistant allele amplified after approximately 36 minutes at 0.8mM dNTPs compared to 22 minutes with 1.2mM dNTPs. A small increase in the difference in amplification time between resistant and susceptible alleles was observed with lower dNTP concentration; 11 minutes compared to 14 minutes using 0.8mM dNTPs. Amplification was unsuccessful with reduced enzyme concentration.

Results showed an increased difference in the timing of amplification between resistant and susceptible alleles at lower $MgSO_4$ concentrations. Susceptible allele controls amplified five minutes later than resistant alleles at 8mM compared to 11 minutes later when the $MgSO_4$ concentration was reduced to 4mM or below (Table 3.4). The time to amplification of the resistant allele control also varied between runs however, this did not appear to be correlated with $MgSO_4$ concentration.

The addition of a denature step prior to amplification decreased amplification specificity. Denaturing template DNA alone and template DNA with primers both resulted in non-specific amplification of all samples tested.

3.4.5 Quantification

Figure 3.9 shows amplification of titrated gDNA control samples, extracted from individual adult worms. Samples amplified in the order of resistant allele frequency, with the exception of the high resistant allele frequency samples (90% and 95%) which amplified at approximately the same time. Highly dilute resistant alleles (5%) and the BZ-susceptible control DNA samples did not amplify, indicating specific amplification and suggesting a sensitivity level of around 10%.

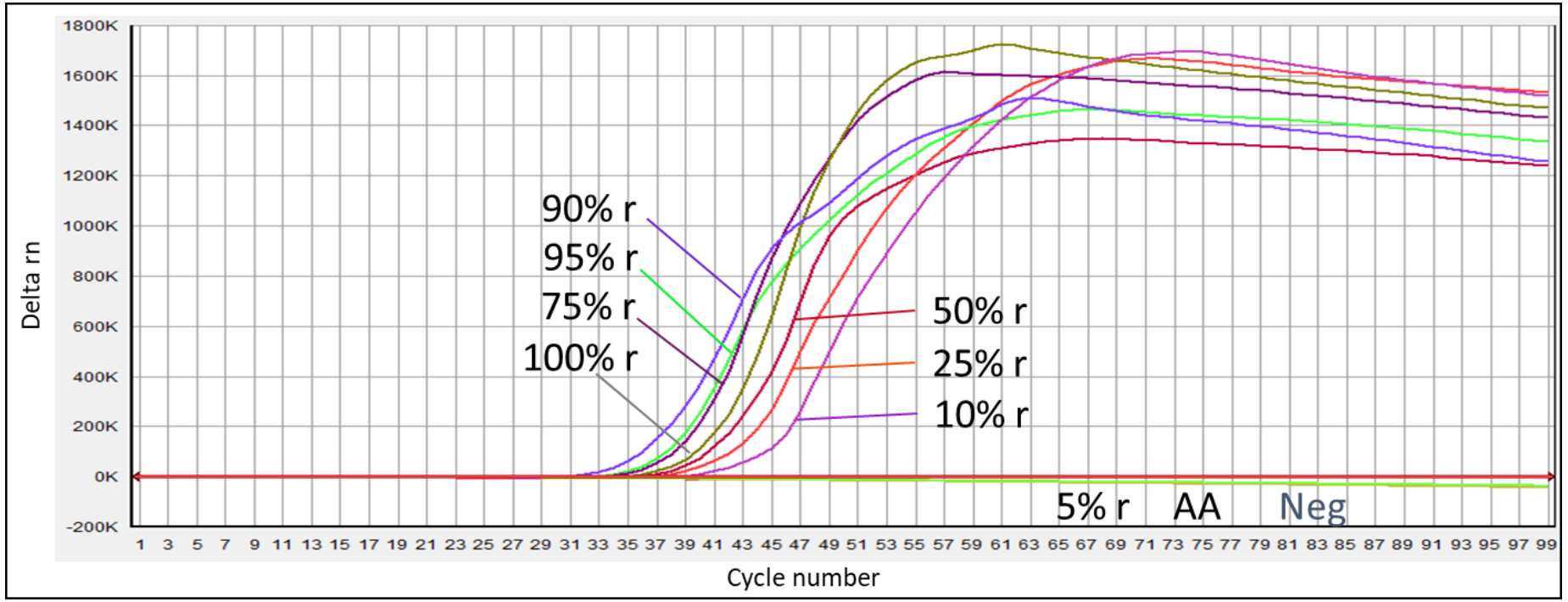


Figure 3.9. Quantification test of prototype LAMP assay.

LAMP assay result showing quantification of resistant allele frequency from titrated gDNA mixtures of ranging from 0 to 100% resistant allele frequency (% r).

A second titration was performed using plasmid DNA samples (Figure 3.10). The prototype assay successfully amplified samples in the order of concentration of the resistant plasmid; from 1ng/µl down to 10 pg/µl between 23 and 38 minutes (Figure 3.10). The susceptible plasmid DNA samples also amplified however, these amplified slower than the resistant plasmids (beginning at ~39 minutes), with the exception of 1pg/µl resistant plasmid which amplified later than the susceptible plasmid at concentrations above 125pg/µl. Differentiation between resistant allele concentrations was evident however, the time difference between resistant allele frequencies was small; approximately seven cycles between 1ng/µl and 125pg/µl which was 3.85 minutes.

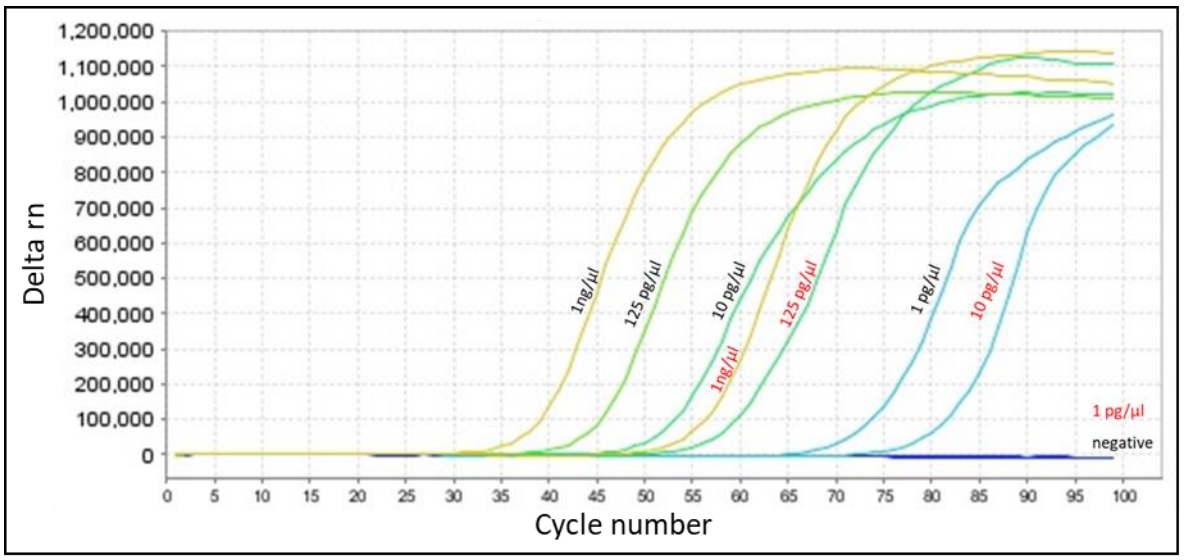


Figure 3.10. Quantification test of prototype LAMP assay using plasmid DNA.

LAMP assay result showing quantification of F200Y allele frequency from titrated plasmid DNA, concentration of the plasmid DNA highlighted; resistant allele (black) and susceptible allele (red).

3.4.6 Application of assay using field samples

The BIP4 prototype assay was tested using crude DNA lysates from individual eggs from field samples previously genotyped by pyrosequencing (Figure 3.11).

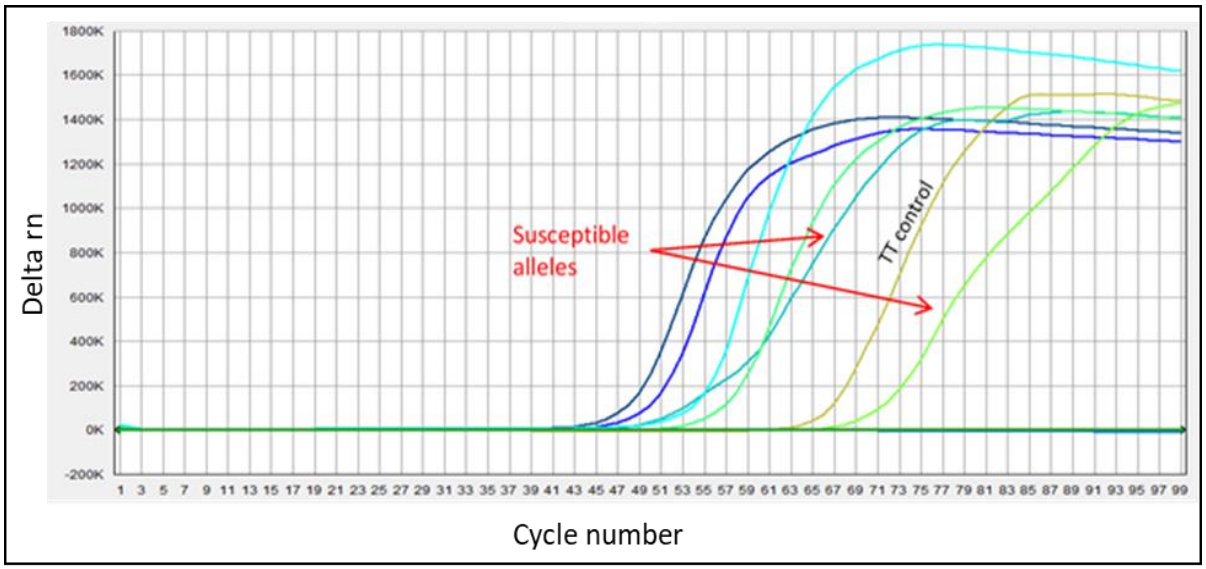


Figure 3.11. Test of prototype LAMP assay with DNA extracted from field samples.

Non-specific amplification of individual DNA lysates from eggs of varying F200Y genotype. Red arrows indicate the amplification of susceptible alleles before and after the resistant allele control DNA sample, un-labelled lines represent amplification of samples previous genotyped as homozygous or heterozygous resistant.

Results indicated non-specific amplification of susceptible alleles at a similar timepoint as samples with resistant genotypes. This was replicated several times with a total of 25 individual egg and L_3 lysates (20 previously genotyped as susceptible, two homozygote resistant and four heterozygote resistant) to ensure that the observed false positives were not due to contamination of previously analysed lysates. A total of 11 samples previously genotyped as homozygous susceptible amplified between 25 and 45 minutes and 6 resistant alleles amplified between 23 and 35 minutes. Results were similar between replicates with several false positives amplified in each reaction.

Amplification of DNA lysates prepared from pools of eggs/L_3 from previously genotyped populations produced similar inconsistent results (Figure 3.12). Pooled lysates did not amplify in order of their previously calculated F200Y allele frequency, the fully susceptible (0% resistant alleles) and low allele frequency (2% resistant alleles) samples were not expected to amplify however, did so quicker than the population previously found to have 32% F200Y resistant allele frequency.

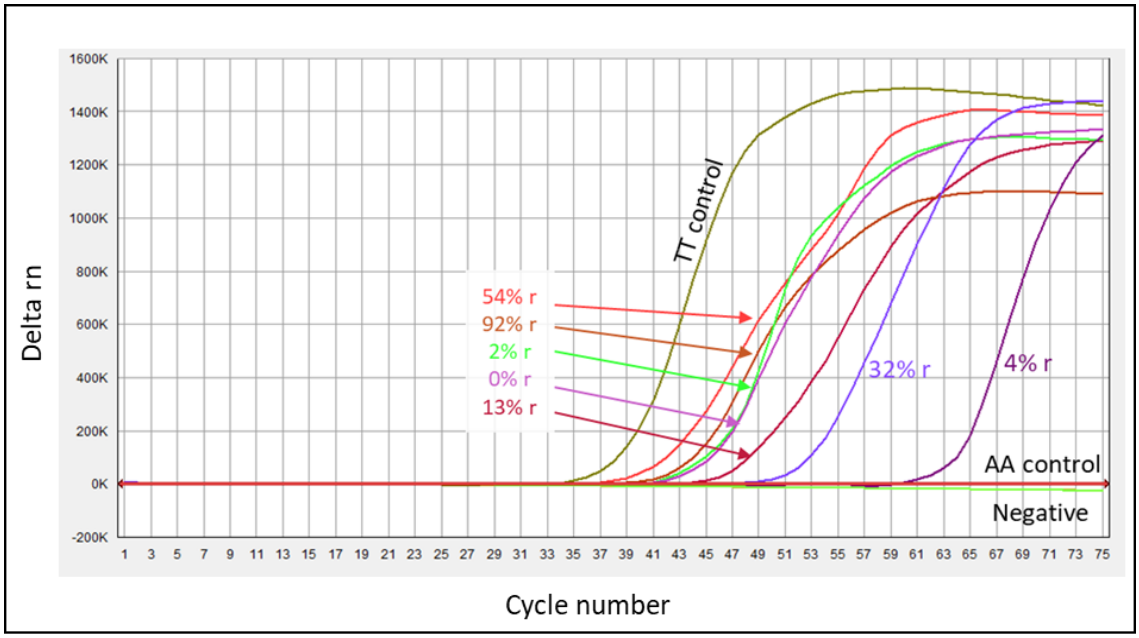


Figure 3.12. Test of prototype LAMP assay with DNA extracted from pooled eggs/L_3.

Non-specific amplification of pooled lysates from eggs/L_3 from farm populations analysed during the genotyping study with F200Y resistant allele frequency (%r).

The reaction was repeated using gDNA from pooled L_3 extracted using the same method as individual adult gDNA control samples (section 3.3.1) previously used for preliminary testing (Figure 3.13) to assess the influence of DNA extraction method on reaction specificity.

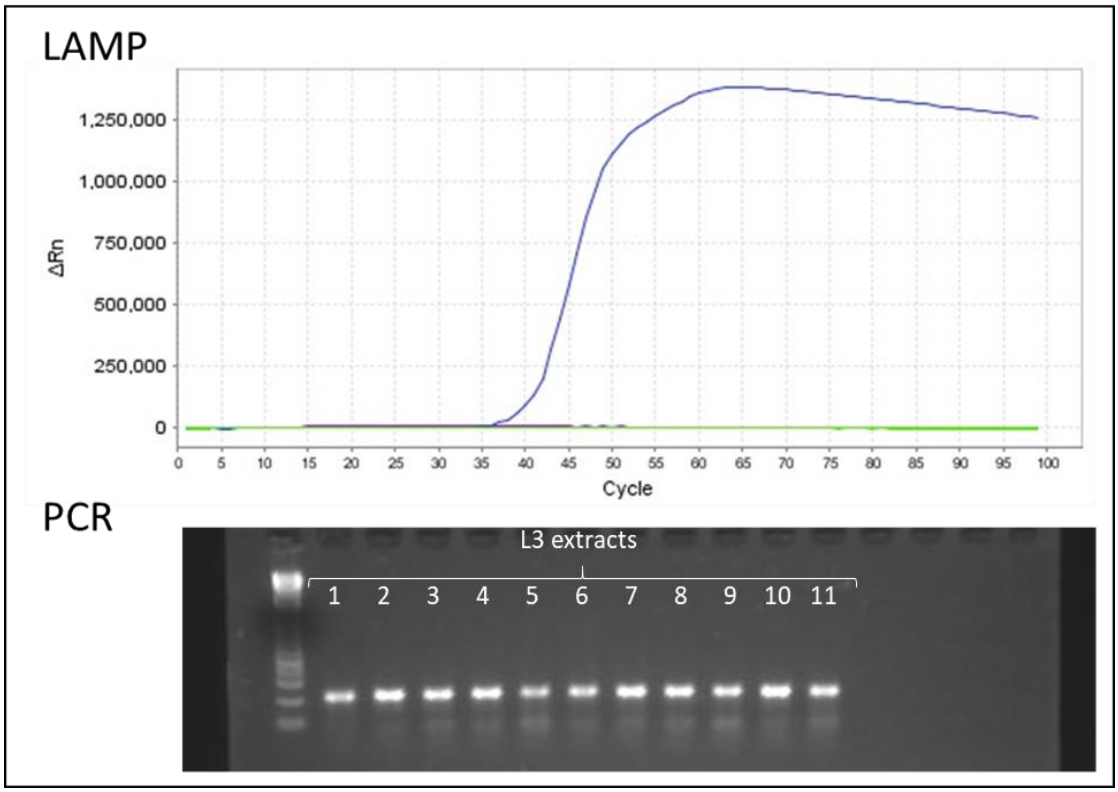


Figure 3.13. Test of prototype LAMP assay with DNA extracted from pools of 100 L_3.

Amplification of gDNA extracted from pools of 100 L_3 of varying expected resistant allele frequency (see table 1 for expected F200Y frequency). Neat and diluted (1:100) DNA extracts were tested using the BIP4 prototype LAMP assay, the single amplification trace is from the positive control; gDNA extracted from one BZ-resistant adult N. battus*. PCR reaction was carried out using neat DNA from L_3 extracts and shows clear amplification in all samples.*

Pooled gDNA extracts from larvae (100 L_3 per sample) of varying resistant allele frequency, all amplified by conventional PCR to produce clear bands but failed to amplify by LAMP (Figure 3.13). The PCR result confirms the presence of amplifiable DNA in the samples. Neat and diluted (1:100) DNA extracts were included in the LAMP assay however, only the resistant gDNA control sample amplified in each reaction (Figure 3.13).

Overall the prototype BIP4 assay provided inconsistent results when tested using field derived samples of eggs and L_3 regardless of the DNA extraction method.

3.5 Discussion

The current proof-of-concept test explored the use of LAMP to detect and quantify the F200Y SNP in *N. battus* populations by selective amplification of the resistant allele. The current prototype assay provided accurate detection of the F200Y resistant allele within ten minutes using plasmid and high quality gDNA extracted from adult parasites. The output of the prototype assay was also found to be semi-quantitative, eluding to a greater diagnostic potential than simple presence/absence. Unfortunately, translation of the test to amplify gDNA lysates extracted from pooled eggs and larvae proved difficult, giving unreliable results. The cause of the failure to amplify pooled field samples remains unclear, possibly due to the quality of DNA extracted or the presence of an unidentified inhibitor. More research is therefore required to determine why the specificity of the test was reduced when amplifying field-derived material.

Six LAMP primer sets were designed and tested in the current study, primer sets BIP1-3, 5 and 6 were discounted at various stages of evaluation due to non-specific amplification or inconsistent results. Primer set BIP4 provided specific, repeatable results during initial screening and so, was retained for optimisation of the prototype assay. The design of suitable discriminating primers for SNP genotyping was challenging due to the ability of some primers to bind successfully in the presence of single nucleotide changes in the binding site sequence. The BIP 4 primer, which placed the SNP on the final nucleotide base of the B2 section of the BIP primer, following the design used by Badolo *et al.* (2012), afforded the most specific amplification of the resistant allele. This finding is in agreement with previous studies which concluded that placement of the SNP site at the 3' end of the primer provided greater specificity compared to 5' or internal sites (Okayama et al., 1989; Simsek and Adnan, 2000).

The correlation between SNP frequency and phenotypic resistance has not been studied for BZ-resistance in *N. battus*. Previous studies suggest the threshold for phenotypic

anthelmintic resistance may be between 15-30% resistant allele frequency in other GIN species (Santos et al., 2017; von Samson-Himmelstjerna et al., 2009). However, this is difficult to measure due to the interplay between multiple SNPs which have been suggested to confer different levels of phenotypic resistance (Kotze et al., 2012) and the likelihood that phenotype/genotype correlation may differ between species and isolates. The prevalence study in chapter 2 found that the F200Y mutation was at a low frequency overall but was present in a large proportion of the *N. battus* populations tested. The high prevalence of the resistant allele reduces the usefulness of a presence/absence test as a large number of populations would test positive when the resistant allele frequency was too low to cause clinical drug failure. Although the continued use of BZ-compounds in this case may lead to an increase in resistant allele frequency over time, use of this drug class early in the season would still significantly reduce the *N. battus* burden in lambs whilst avoiding additional selection pressure for other anthelmintic classes required later in the season. Quantification of the mutation would therefore provide a more meaningful estimation of the phenotypic efficacy upon which to potentially base treatment decisions. Several quantitative LAMP assays have been reported, estimating template concentration based on time to amplification (Abbasi et al., 2016; Drame et al., 2014) or detection of an electrochemical signal monitoring the intercalation of DNA-binding reporter molecules (Hsieh et al., 2012). Quantification in the current experiment was measured by time to amplification. The prototype BIP4 assay provided semi-quantitative results using both titrated gDNA and plasmids; amplifying samples in the order of resistant allele concentration down to 10 pg/µl. Quantification by amplification time relies on 'gating' consistent results i.e. categorising resistant allele frequency based on ranges of time to amplification. The prototype assay provided significant separation between the amplification time of resistant and susceptible samples at 1ng/µl, sufficient for a robust presence/absence differentiation (15 minutes). However, the separation in amplification time between resistant allele frequencies was

minimal; less than 4 minutes difference in amplification time was observed between samples with an eight-fold difference in resistant allele frequency. The results of the gDNA and plasmid titrations indicate that quantification may be possible however, separation between resistant allele frequencies would have to be increased. Whilst the current results were similar to some those previously published (Soleimani et al., 2013), the linear relationship observed in other studies in which ten minutes difference in reaction time was equivalent to a ten-fold difference in template concentration (Hsieh et al., 2012) should be aspired to in order to provide a robust, quantitative diagnostic tool.

To be of use as a diagnostic test, the template material must be extracted from an accessible life stage; ideally DNA extracted from eggs which could be collected from faeces. Amplification from crude DNA preparations has been demonstrated using LAMP (Edwards et al., 2014; Kaneko et al., 2007) and would reducing labour, technical expertise and equipment requirements of the test. Analysis of pooled samples would also be beneficial, allowing representative analysis of a flock from a single reaction. However, translation of the assay to lysates and gDNA extracted from field-derived eggs and L_3 proved challenging in the current study. Reactions amplifying lysates prepared from individual and pooled eggs and L_3 repeatedly amplified susceptible and resistant alleles at approximately the same time. Amplification of both resistant and susceptible samples is acceptable however, a consistently significant difference in the time of amplification between susceptible and resistant alleles would be critical in order to reliably interpret results. The stringency and reliability of the current prototype assay when amplifying field samples was not sufficient for a diagnostic assay. The reason for differential results obtained from analysis of different *N. battus* life stages remains unclear.

The assay design followed the optimal LAMP conditions set out in the literature (Notomi et al., 2000) with the exception of the relative melting temperatures of the inner and outer

primers which may have resulted in a small amount of initial amplification from the outer primers which were not discriminative and may be responsible for non-specific amplification. The distance between primer sites and secondary structures of the DNA must be considered during LAMP primer design given the lack of denaturing steps, usually primer sites could be altered to fulfil optimised conditions. However, the placement of the SNP limits the options for primer design. Sample concentration and DNA extraction method did not appear to influence the specificity of amplification from eggs and L_3 (section 3.4.6) and no differences were observed in DNA sequence between life stages of *N. battus* (data not shown). Variation in specificity of amplification may be due to the quality or quantity of DNA template present in the sample. Both plasmid and purified gDNA from adult nematodes, which provided consistent results, were high quality templates. Despite dilution of the genomic and plasmid DNA to similar concentrations, plasmid samples contain a greater number of copies of the target gene compared to whole nematode gDNA samples and thus may explain amplification of the susceptible allele using plasmid but not gDNA templates. By contrast, DNA lysates from individual eggs/L_3 provide a low concentration, poor quality template which was found to produce inconsistent results and false positives with the prototype BIP4 assay. Despite the wide application of LAMP technology both in lab-based and POC diagnostics, the method has been repeatedly found to lack specificity (Gadkar et al., 2018; Gray et al., 2016). In the case of BZ-resistance in *N. battus*, a false positive result could mistakenly determine that a population was resistant to BZ compounds when treatment failure could be due to another factor such as incorrect dosing technique. Several modifications have been published to overcome specificity issues including the use of peptide nucleic acid probes, designed to bind wildtype sequences to prevent amplification (Minnucci et al., 2012). False amplification has frequently been attributed to post-amplification processing and non-specific detection methods such as turbidity readings or intercalating dyes. One method of overcoming specificity problems is the use of sequence-specific detection methods rather than specific

amplification and several such solutions have been published. Each method amplified the target region using LAMP then detected the SNP within the product using a variety of methods including binding Au-nanoprobes and testing for aggregation (Veigas et al., 2013), self-quenching/dequenching probes attached to loop primers which fluoresce upon binding the resistant allele sequence (Gadkar et al., 2018) or fluorescent cationic probes from which the sequence can be determined from the colour of the DNA:probe complex precipitate (Mori et al., 2006). The later method has the potential for both multiplexing and translation into a point of care device, development of similar tests for livestock pathogens could have wide ranging applications including anthelmintic resistance screening or quarantine testing. We have demonstrated in the current study that LAMP is capable of amplifying DNA from *N. battus*, redesigning the assay to place the SNP between primer sites and using probes for the detection of SNPs or the prevention of amplification of the susceptible allele may provide a more robust diagnostic assay than selective amplification. Perhaps also allowing for multiplexing to test for the other SNPs associated with BZ-resistance in ovine nematodes. Serval novel isothermal nucleic acid amplification technologies (iNAATs) have been described in recent years. Methods such as helicase-dependent amplification (HDA), Rolling circle amplification (RCA) and smart amplification 2 (SMAP2) each utilise different denaturation methods and polymerase and as such, have different strengths and weaknesses. SNP detection has been demonstrated using RCA (Faruqi et al., 2001) and SMAP2 (Mitani et al., 2007). SMAP2 was based on LAMP principles however, the primer sets are designed to amplify asymmetrically which minimises mis-priming, a major issue in SNP-detection LAMP. A study has been published reporting a set of SMAP2 assays for the detection of the F167Y, E198A and F200Y SNPs associated with BZ-resistance in the human hookworm *Necator americanus* (Rashwan et al., 2016). The assay showed highly sensitive and specific amplification of the resistant alleles at each SNP from control and faecal-derived material, providing presence/absence results in approximately 40 minutes. Re-designing these

primers to amplify the same regions in *N. battus* may provide a set of rapid tests for BZ-resistance SNPs however, to my knowledge, SNP quantification has not yet been demonstrated using this technology and as previously discussed, the demand for a presence/absence test for BZ-r SNPs in *N. battus* may be limited.

For significant uptake in the livestock industry, the test for resistance must be non-invasive and the template easily extracted from an accessible and appropriate parasite life stage e.g. eggs in faeces. Currently, two hurdles remain; firstly, the inability to extract DNA from *N. battus* eggs in the field and secondly, different iNAAT technologies are currently incapable of providing quantitative results (Rashwan et al., 2016).

Nematode eggs, particularly *N. battus,* possess a hard, external shell which protects the egg from adverse environmental conditions on pasture therefore, DNA extraction from nematode eggs is a major hurdle for the detection of parasitic diseases. Cracking *N. battus* eggs for DNA extraction in the laboratory requires eight rounds of freezing with liquid nitrogen and thawing at 100°C prior to proteinase K digestion (chapter 2), a method which is not readily suited for translation to the field at the moment, albeit that there have been recent developments in the area of 'in-field' bead-beating extraction tools (TerraLyzer, Zymo Research, USA). Perhaps DNA extraction from *N. battus* eggs in the field could be revisited in the future.

It may be possible to translate versions of the current prototype primer set to a different iNAAT technology or alter the primers published by Rashwan *et al.* (2016) for detection of the F200Y SNP in *N. battus*. However, the results need to be quantitative, whereas, currently, they are not. As discussed previously, a meaningful output from the test would have to be at least semi-quantitative to prevent identification of extremely low resistant allele frequencies from the majority of populations. Due to the importance of quantification and the inability to develop an on-farm test given current DNA extraction technologies, the next

logical step was to investigate alternative methods which could be used in a diagnostic lab setting.

Genomic sequencing technologies have undergone significant advancement in recent years both in whole genome and targeted approaches, making these platforms more accessible for diagnostic use with reduced costs and greater resources. Deep amplicon sequencing using an Illumina MiSeq platform may be suitable for the detection and quantification of SNP mutations from pooled samples, providing a high-throughput alternative to pyrosequencing. Deep amplicon sequencing may also maximise the information gained from each sample, providing sequencing information which could be used to explore haplotype diversity and population dynamics as well as study-wide screening for additional, unknown mutations. This technology has been applied in the veterinary parasitology field with Illumina MiSeq assays having recently been developed to identify both trichostrongylid species present and BZ-resistant allele frequency from pooled samples within a single run (Avramenko et al., 2018); a holy grail for livestock parasitologists. For those reasons, the remaining time was allocated to the development and evaluation of the MiSeq assay discussed in chapter 4 rather than the continued development of LAMP with sequence-specific detection or evaluation of other rapid diagnostic platforms such as SMAP2 or RCA.

3.6 Conclusion

Six primer sets were designed and evaluated for the detection and quantification of the F200Y SNP in the β-tubulin isotype 1 gene which confers BZ-resistance in *N. battus*. One primer set (BIP4) was selected for further optimisation. The prototype assay selectively amplified the resistant allele when tested using gDNA samples extracted from single adult parasites and consistently amplified the resistant allele ten minutes earlier than the susceptible allele when tested using plasmid DNA. However, when applied to field-derived samples, the prototype assay produced unreliable results. SNP detection has been

demonstrated using alternative isothermal rapid amplification technologies. However, the motivation to develop rapid, isothermal assays was to translate diagnosis to a field-based setting. Diagnosis of *N. battus* in the field remains unfeasible at present due to the DNA extraction method required to break the tough, outer shell of the eggs, thus alternative laboratory-based methods such as next generation sequencing were explored instead. Given future development of robust DNA extraction methods suitable for *N. battus* eggs in the field then the alternative rapid diagnostic methods discussed here may be of use given further development.

4 Comparison of next generation deep amplicon sequencing and pyrosequencing technologies in the detection and quantification of benzimidazole resistance in UK *N. battus* populations

4.1 Abstract

Knowledge of the genetic mutations associated with benzimidazole resistance creates the opportunity to develop molecular tools capable of assessing the frequency of resistant alleles from a single sample. Currently, molecular detection relies on pyrosequencing analysis of individual parasites which is a labour-intensive process. The current study explored the use of deep amplicon sequencing using an Illumina MiSeq platform as a high-throughput alternative to pyrosequencing. A MiSeq assay was designed to identify three SNPs in the β-tubulin isotype 1 gene associated with BZ-resistance (F167Y, E198A and F200Y) in *N. battus* from pooled samples. The aim of the current study was to detect and quantify BZ-resistance SNPs in *N. battus* populations collected from throughout the UK using deep amplicon sequencing and compare the results with those obtained from the pyrosequencing assay in chapter 2. A total of 214 populations from 174 farms were included in the deep amplicon sequencing, of those, 201 populations from 161 farms were previously analysed by pyrosequencing and were included in the comparison. Pooled DNA extracts from 500-1000 parasites were sequenced by MiSeq. Results from the two technologies were comparable at the F200Y locus (r^2 = 0.96) however, inconsistencies were identified at low allele frequency. The results for the F167Y SNP were variable between the assays however, this is likely due to the low inter-population resistant allele frequency at this locus (0-13%). No SNP mutations were identified at codon 198 by either pyrosequencing or deep amplicon sequencing. Despite low level variation between the results obtained by MiSeq and pyrosequencing, either method could be used as a diagnostic tool to test for BZ-resistance following suspected treatment failure.

4.2 Introduction

Knowledge of the molecular mechanism of BZ-resistance opens up the opportunity to use molecular diagnostic tools for the detection and quantification of these mutations. Three single nucleotide polymorphisms (SNPs) in the β-tubulin isotype 1 gene have been repeatedly identified as conferring BZ-resistance in a range of nematode species, including *N. battus*. Molecular diagnostics have several benefits over the traditional faecal egg count reduction test, primarily, the ability to test a single sample collected from untreated animals, providing rapid results. There is currently no 'gold-standard' method for molecular diagnosis of anthelmintic resistance. Pyrosequencing assays have been developed for the detection and quantification of SNPs associated with BZ-resistance in a number of GIN species including *T. circumcincta* (Skuce et al., 2010), *H. contortus* (von Samson-Himmelstjerna et al., 2009) and *T. colubriformis* (Ramunke et al., 2016). The pyrosequencing assays have been used in research, particularly in genotyping surveys to create a picture of the status of BZ-resistance in different countries and GIN species (Ramunke et al., 2016; Redman et al., 2015; von Samson-Himmelstjerna et al., 2009).

Recent advances in sequencing technology have made these techniques more accessible for both research and diagnostic usage. Deep amplicon sequencing methods such as Illumina MiSeq are highly versatile and provide a wealth of data, analysing up to 384 populations within a single run. The wide-ranging application of next generation sequencing techniques rely on the development of analysis pipelines to manipulate and analyse the high volume of sequencing data produced during each run. Recent applications for veterinary nematodes include nemabiome analysis; the study of nematode species from pooled faecal samples (Avramenko et al., 2015) and the detection of SNPs associated with BZ-resistance in *T. circumcincta* and *H. controtus* (Avramenko et al., 2018).

The aim of the present study was to evaluate deep amplicon sequencing for the detection and quantification of SNPs associated with BZ-resistance in *N. battus* and compare this novel technique with the gold-standard pyrosequencing assay used in chapter 2.

4.3 Materials and Methods

Sample collection and *N. battus* egg extraction protocols are detailed in chapter 2.

4.3.1 Parasite material and DNA extraction

A total of 214 *N. battus* populations were included in the deep amplicon sequencing, pools of 500-1000 ethanol-fixed parasites were used per population. Eggs/L_3 were centrifuged at 16000g for 4 minutes and the supernatant removed, the parasite material was then washed three times in worm lysis buffer (50mM KCl, 10mM Tris (pH 8.0), 2.5mM MgCl2, 0.45% Nonidet p-40, 0.45% Tween-20, 0.01% gelatin) to remove the ethanol, resulting in a final volume of 150µl. Following the final wash step, samples containing eggs were subjected to 8 cycles of freeze/thaw (30 seconds liquid nitrogen/1 minute at 100°C) to crack the outer shell of the egg for enzyme digestion. Proteinase K (Promega, USA) was added to each sample to provide a final concentration of 0.8mg/ml. Samples were incubated on a heatblock at 56°C overnight for digestion to take place, the temperature was then increased to 95°C for 10 minutes to deactivate the proteinase K enzyme. Lysates were cleaned using the quick DNA extraction kit (Zymo, USA) following the manufacturers protocol and extracted DNA was eluted in 1xTE buffer (Sigma, USA) to stabilise DNA for transport to University of Calgary, Canada for sequencing.

4.3.2 Amplification of the target sequencing region and preparation of the sample library for sequencing

4.3.2.1 Target region amplification

The region of interest; β-tubulin isotype 1 gene, exon 4-5, was amplified by PCR prior to sequencing to increase the copy number of the sequence of interest and to attach Illumina adapter sequences, providing annealing regions for sequencing primers downstream. The adapter sequences were separated from the primer sequence by 0-3 universal 'N' nucleotides to introduce sequence variation for MiSeq analysis. PCR reactions were performed using Kappa HiFi Hotstart PCR kit (Kappa Biosystems, Switzerland) and were set up on ice as follows; 5µl 5X buffer, 10mM dNTP mix, 10µM *N. battus* forward primer + adapter sequence, 10µM *N. battus* reverse primer + adapter sequence, 0.5U Kappa HiFi hotstart polymerase, 4-10µl of template DNA and molecular grade water; 25µl final reaction volume (primer sequences in Table 4.1). Reactions were incubated at 95°C for 2 minutes followed by 30 cycles of 98°C for 20s, 62°C for 15s and 72°C for 15s, final extension phase at 72°C for 2 minutes. PCR products were run on a 2% agarose gel using SYBR green loading dye to confirm amplification. PCR was repeated for samples which failed to amplify or produced only a faint product band using up to 10µl template and increasing the number of cycles in the PCR to 35.

Table 4.1. N. battus-specific PCR primers used to amplify the β-tubulin isotype 1 gene exon 4-5.

Primer	Sequence
Forward	ACKCATTCWCTTGGAGGAGGC
Reverse	GTSAGTTTCAATGTTCGGAAACAG

4.3.2.2 PCR product clean up

Magnetic bead purification was performed to remove unused primers, dNTPs and contaminants. Briefly, 20µl PCR products were mixed with 25µl AMPure XP beads (Beckman Coulter, USA) and incubated at room temperature for 5 minutes to allow DNA to adhere to the magnetic beads. The 96-well plate was then placed on a magnetic stand (Applied Biosystems, USA) for 2 minutes to allow beads to concentrate at the side of the well and the supernatant was removed. The beads were washed twice with 80% EtOH; with the plate on the magnetic stand, 200µl of EtOH was added to each well and incubated for 30 seconds, the supernatant was then removed and discarded. Following the second EtOH wash, beads were air-dried for 15 minutes to allow remaining EtOH to evaporate. The plate was removed from the magnetic stand and beads were re-suspended in 40µl of molecular grade water. Following a 2 minute incubation at room temperature, the plate was placed on the magnetic stand and 32µl of purified DNA was removed into a new 96-well plate.

4.3.2.3 Population barcoding

Illumina barcodes were added to the purified PCR products using a second-round amplification. Unique combinations of barcoded primers were added to each PCR reaction to allow for sample identification after sequencing, using primer set Nextera XT index kit V2 set (Illumina, USA) see appendix 2 for primer sequences. PCR reactions were performed using Kappa HiFi Hotstart PCR kit (Kappa Biosystems, Switzerland) and were set up on ice as follows; 5µl 5X buffer, 10mM dNTP mix, 10µM forward primer (S502 – S522), 10µM reverse primer (N701 – N729), 0.5U Kappa HiFi hotstart polymerase, 3µl of first round PCR product and molecular grade water; 25µl final reaction volume. Reactions were incubated at 98°C for 45s followed by 7 cycles of 98°C for 20s, 62°C for 20s and 72°C for 2 minutes. Second round PCR products were cleaned using magnetic bead purification as described in section 4.3.2.2.

4.3.2.4 Library preparation

Individual samples were quantified using a Nanodrop spectrophotometer and samples were pooled to create a normalised library containing approximately 50ng/µl of each sample. The library was then quantified and diluted to 5ng/µl. The resulting library was quantified by qPCR using the Illumina library Quantification Kit and Universal qPCR Mix (Kappa Biosystems, Switzerland). Serial dilutions of the library were prepared in triplicate using 10mM Tris-HCl pH 8.0, 0.05% Tween-20 to achieve 1:2000, 1:4000 and 1:8000 dilutions. Reactions were set up on ice as follows; 12µl Sybr fast qPCR mastermix containing 10X primer premix, 4µl molecular grade water and 4µl library dilutions. Supplied 425bp DNA standards were included as positive controls and molecular grade water as a negative control. Reactions were incubated at 95°C for 5 minutes followed by 35 cycles of 95°C for 30s and 60°C for 45s, fluorescence recorded in the Sybr green channel. The library was quantified using absolute quantification against the supplied DNA standards.

The library was then diluted to 4ng/µl with molecular grade water. 5µl of 4nM library was added to 5µl 0.2M NaOH, vortexed and incubated at room temperature for 5 minutes to denature the DNA. Following incubation, the library was diluted to 15pmol using chilled hybridisation buffer (HT1, Illumina, USA). The PhiX library (Illumina, USA) was also denatured and diluted as described for the sample library, this was included in the sequencing run to increase diversity. The libraries were combined at a ratio of 80% sample library: 20% PhiX library and loaded onto the MiSeq flow cell for sequencing.

4.3.3 Analysis pipeline for Illumina sequence data

Sequence data was generated by MiSeq in FASTq files, proving information on the nucleotide base sequence and a confidence rating for each base called. The first stage of the pipeline converts the FASTq files into a FASTA format whilst retaining the quality information separately. Sequences were then aligned and the Illumina barcode and adapter sequences

removed. At this point, any sequences significantly out with the expected size bracket (200-450bp) were removed. Test sequences were compared to a reference database containing sequence information from all major veterinary GIN, created by sequencing from experimental mono-infections (Avramenko et al., 2015). All sequences classified as *N. battus* were compared to the reference to highlight differences in the sequence. Mutations were then graded by their potential impact; synonymous mutations and those positioned within the intron are of low impact as these mutations will not alter the downstream product of the gene once transcribed; non-synonymous mutations within the exons and conversion to a stop codon may have a significant impact on the transcribed gene (moderate to high impact mutations). The frequency of each moderate and high impact mutation was calculated, those occurring less than 0.1% within the dataset were likely polymerase errors and therefore dropped from the analysis. Remaining mutations were believed to be present in the population and their frequencies were calculated.

4.3.4 Statistical Analysis

Allele frequencies of replicate samples analysed by deep amplicon sequencing were compared using a Kruskal Wallis test and the resistant allele frequencies obtained from pyrosequencing and MiSeq were compared by linear regression analysis. A Fisher exact test was also used to compare the presence or absence of F200Y resistant alleles between deep amplicon sequencing and pyrosequencing results. All analyses were carried out using R version 3.2.5.

4.4 Results

A total of 214 populations from 174 farms were successful analysed using deep amplicon sequencing. Allele frequencies derived from deep amplicon sequencing technical replicates of each SNP were not statistically significantly different from each other (H = 0.128, p>0.05).

4.4.1 Prevalence of F167Y in UK *N. battus* populations

Deep amplicon sequencing identified the F167Y mutation at an allele frequency >1% in 8 of the 214 populations tested, originating from 6 independent farms. The overall frequency of F167Y was low; 0.2±0.07%, range 0 – 10%. A subset of 18 *N. battus* populations were analysed at position 167 by pyrosequencing (chapter 2). Of the 18 populations tested, the F167Y mutation was identified in 4 populations ranging from 3 – 13%. In two populations, the F167Y SNP was identified by deep amplicon sequencing at frequencies of 0.7 and 7.5% but were not detected by pyrosequencing (Table 4.2).

Table 4.2 Mean farm genotyping results from the analysis of codon 167 by pyrosequencing and deep amplicon sequencing.

Method	Number of farms	Number of farms with r allele	Percentage of farms with r allele	Mean r-allele frequency (%)	Range of r-allele frequency
Pyrosequencing	18	4	15	1.1	0-13
Deep amplicon sequencing	216	6	3	0.2	0-10

4.4.2 Prevalence of E198A in UK *N. battus* populations

The BZ-resistance associated SNP E198A was not identified in any of the populations tested by either pyrosequencing or deep amplicon sequencing.

4.4.3 Prevalence of F200Y in UK *N. battus* populations

The F200Y mutation was identified in 22 farm populations throughout the UK. The overall allele frequency was low; (mean ±SEM) 2±0.8%; Table 4.3.

Table 4.3 Mean regional F200Y allele frequency identified by pyrosequencing (pyro) and deep amplicon sequencing (MiSeq) in Scottish, English and Welsh N. battus farm populations.

		Number of farms		**n farms with r allele**		**Percentage of farms with r allele**		**r-allele frequency (%)**		**Range of r-allele frequencies**	
		Pyro	MiSeq	Pyro	MiSeq	Pyro	MiSeq	Pyro	MiSeq	Pyro	MiSeq
All		161	161	37	21	23	8	2.7	2.4	0-92	0-92
Scotland	Overall	81	81	10	2	12	3	1.1	1.1	0-65	0-88
	North	46	46	7	1	15	2	1.8	1.9	0-65	0-88
	South	35	35	3	1	9	3	0.2	0.01	0-4	0-0.4
England	Overall	72	72	25	19	35	26	4.6	4.1	0-92	0-92
	North East	14	14	5	2	36	14	1.3	0.2	0-6	0-2
	North West	15	15	10	13	67	87	18.8	19.0	0-92	0-92
	South central	11	11	1	0	9	0	0.2	0	0-2	0
	South East	8	8	2	1	25	13	0.9	0.2	0-4	0-2
	South West	24	24	7	3	29	13	1.0	0.3	0-6	0-4
Wales		8	8	2	0	25	0	0.7	0	0-3	0

4.4.4 Comparison of deep amplicon sequencing and pyrosequencing results

A total of 201 *N. battus* populations from 161 farms were successfully analysed by both deep amplicon sequencing and pyrosequencing at the F200Y locus. Results were largely comparable between the two technologies (Figure 4.1 - Figure 4.3). Pyrosequencing identified the resistant F200Y mutation in 37 of 161 analysed farm populations with a mean resistant allele frequency of 4.5 ±1% (±SEM) (range 0-92%). Deep amplicon sequencing identified the F200Y mutation in 21 of the 161 farm populations; mean resistant allele frequency 3.8 ±1% (range 0-92%). Figure 4.1 illustrates the agreement between the technologies; deep amplicon sequencing and pyrosequencing agreed at a presence or absence of resistant allele level on 83% of farms. Suspected resistant alleles; where one technology identified the F200Y mutation and the other did not, occurred in 17% of populations, suspect resistant alleles were more commonly identified by pyrosequencing than MiSeq; in 14% and 4% of populations respectively. The resistant allele frequencies detected in suspect resistant populations were low, ranging from 0.4-1.8% resistant alleles in deep amplicon sequencing and 1.7-6% in pyrosequencing. The difference in the observed presence or absence of F200Y resistant alleles between the two tests was found to be statistically significant using a fisher exact test (odds ratio = 1.7; CI = 1.0, 2.9; p=0.046).

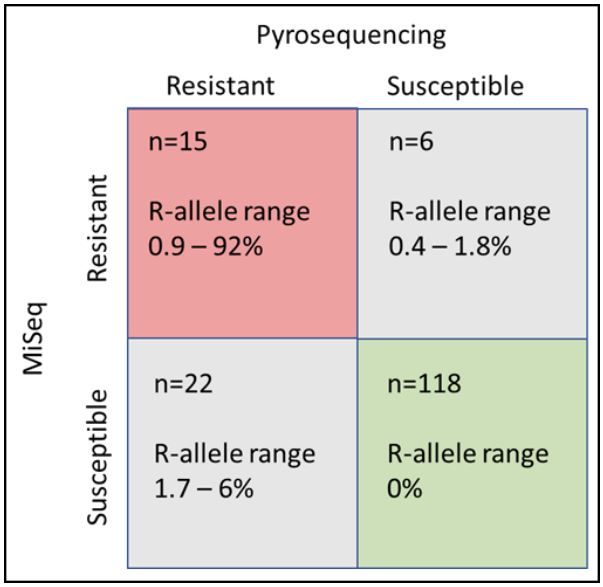


Figure 4.1 Comparison of the results of F200Y analysis between pyrosequencing and deep amplicon sequencing (MiSeq) regarding the presence or absence of resistant alleles and the range of resistant allele frequencies observed.

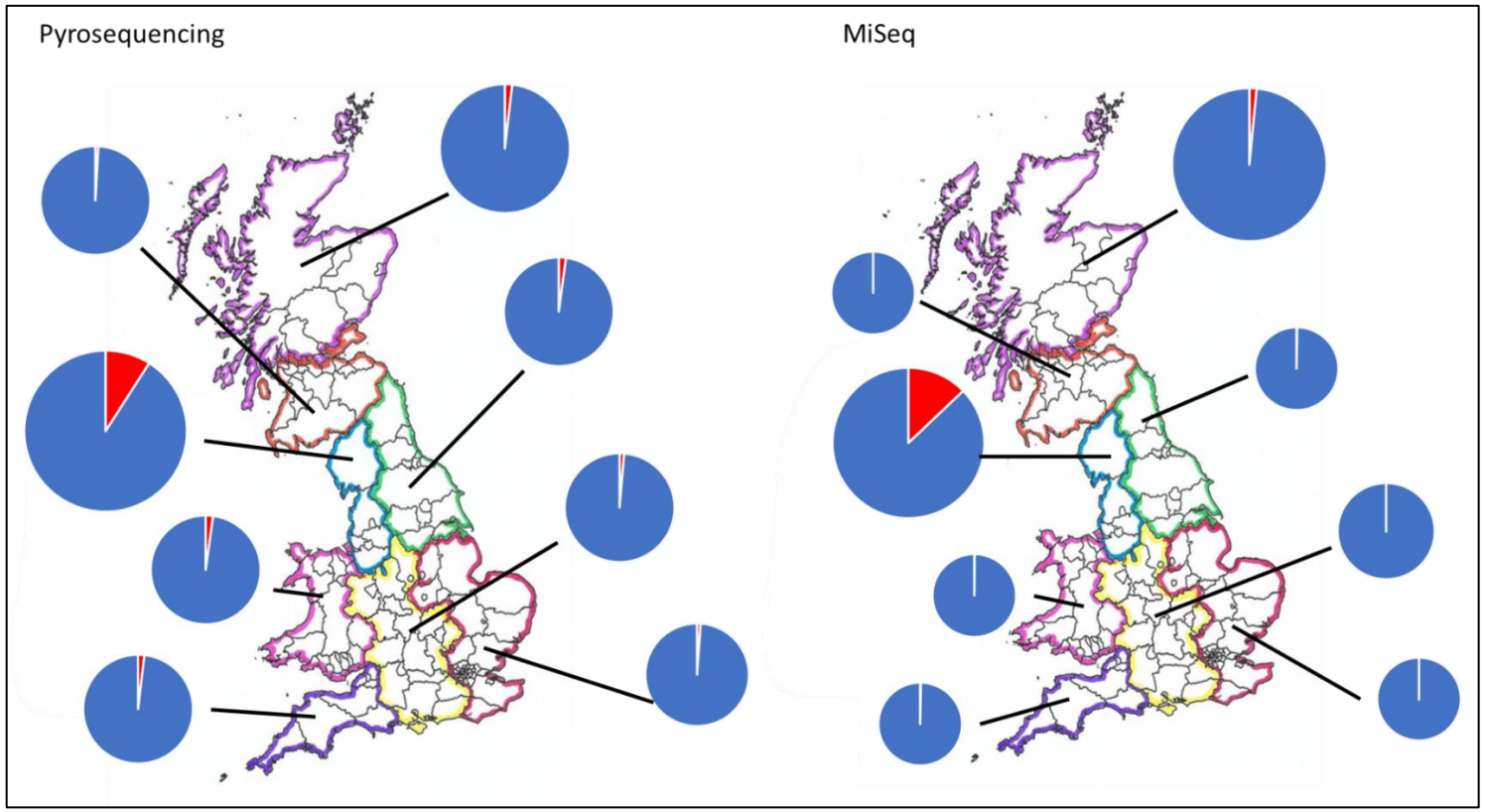


Figure 4.2 Mean F200Y allele frequency by UK region using pyrosequencing and MiSeq analysis.

Susceptible genotypes are represented in the pie charts in blue and resistant in red.

Figure 4.2 illustrates the distribution of the F200Y resistant allele between UK regions by pyrosequencing and deep amplicon sequencing. As described in chapter 2, binomial logistic regression analysis of the distribution of F200Y resistant alleles identified North West England as possessing a statistically significantly higher F200Y resistant allele frequency compared to other regions of the UK. The F200Y SNP was identified in a greater number of regions by pyrosequencing compared to deep amplicon sequencing however, deep amplicon sequencing was in agreement with pyrosequencing results in that it identified resistant alleles in the regions which were highlighted as confirmed or suspected focal regions of resistance; North West England and North Scotland respectively.

Comparison of the pyrosequencing and deep amplicon sequencing F200Y allele frequencies by linear regression found significant agreement between the results. Analysis indicated that 96% of the variance in deep amplicon sequencing was explained by the model ($F(1, 159) = 4128$, $p < 0.001$) with a R^2 of 0.963. The resistant allele frequency obtained by deep amplicon sequencing increased significantly with that obtained from pyrosequencing ($\beta = 0.9$ (95%CI = 0.87, 0.92), $p < 0.001$). Figure 4.3 illustrates the variation between the methods, indicating that no single platform was consistently over- or under-estimating resistant allele frequency. The overall mean difference between the methods was close to zero and with the exception of a small number of anomalies, the variation in resistant allele frequency was within a standard deviation of the mean difference, i.e. within the acceptable limits of the test.

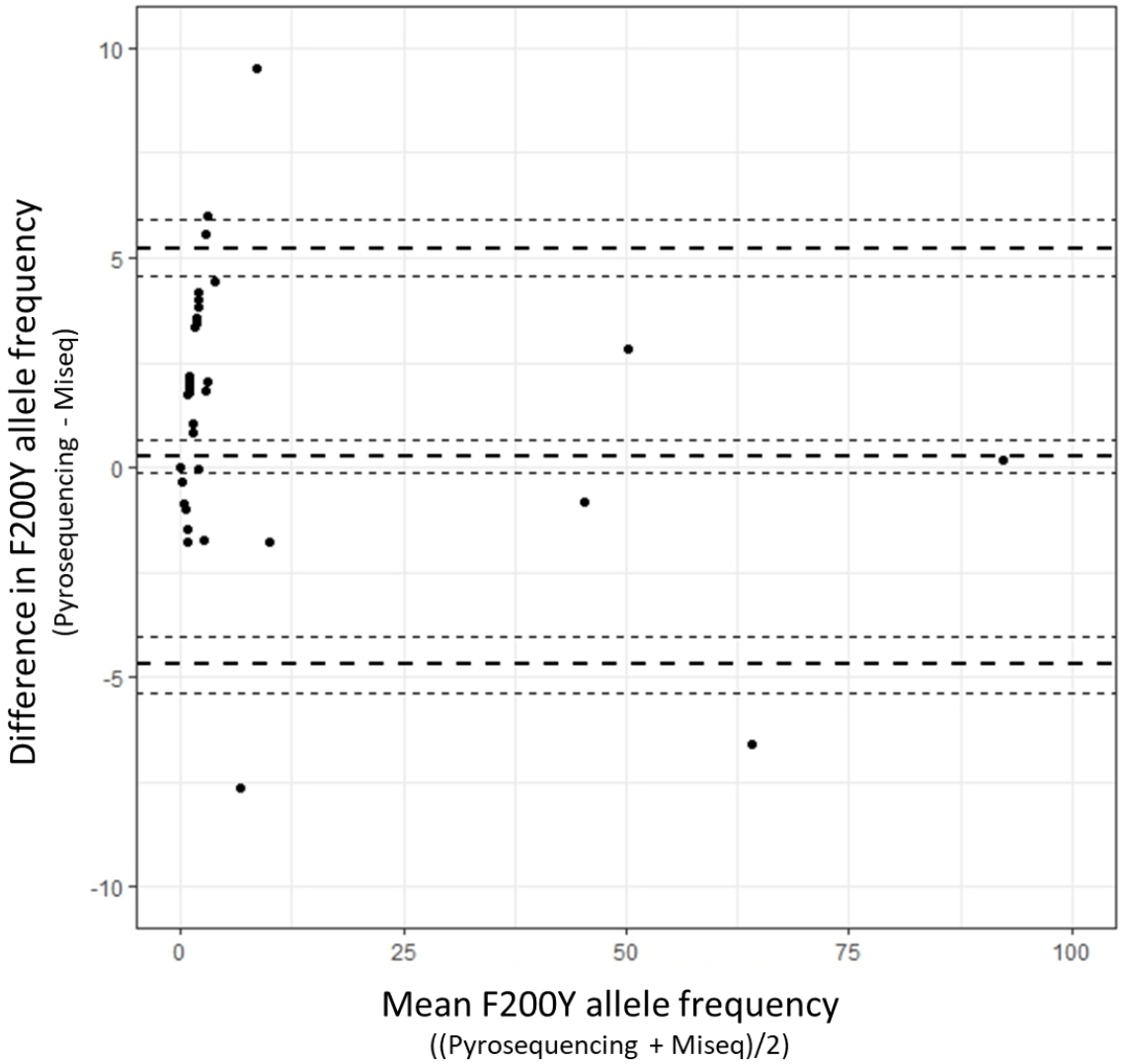


Figure 4.3. Bland Altman comparison of F200Y allele frequency results obtained from pyrosequencing and deep amplicon sequencing (MiSeq).

The difference in F200Y allele frequency obtained from pyrosequencing and deep amplicon sequencing plotted against the mean F200Y allele frequency from each platform. The dashed lines (---) represent the mean difference in results between the two methods and ±1.96 standard deviation of the difference, with the 95% confidence intervals of each indicated by the dotted lines (…).

4.5 Discussion

There is currently no 'gold standard' method for detecting and measuring SNP mutations. However, in the current study we demonstrated that both pyrosequencing and deep amplicon sequencing are equally suitable methods for analysing SNPs. Results were largely comparable between the technologies when analysing the F200Y locus, frequencies were more varied between the platforms at low resistant allele frequency.

The results obtained using pyrosequencing and deep amplicon sequencing targeting the F200Y mutation were statistically comparable by linear regression. Variation in F200Y allele frequency between the two technologies occurred largely at low resistant allele frequency. The technologies agreed on the presence or absence of the F200Y SNP in 83% of the populations tested however, 17% were suspected to contain the resistant allele as it was detected by only one assay. Resistant alleles were identified in 22 populations by pyrosequencing and not deep amplicon sequencing (F200Y range 1.7 - 6%) and a further six populations by deep amplicon sequencing and not pyrosequencing (F200Y range 0.4 - 1.8%). Low level variation was expected as pyrosequencing and deep amplicon sequencing were not performed on the same individual parasites but different aliquots from a large population.

A similar level of inconsistency was identified between pyrosequencing and deep amplicon sequencing results in a previous study of benzimidazole resistance in strongyle nematodes (Avramenko et al., 2018). Both studies have illustrated higher resistant allele frequency in either technology therefore, it can be assumed that neither assay is consistently over-estimating the resistant allele frequency. There are several possible explanations for the variation reported including testing of different individuals within a large population, sequencing errors or bias, un-detected contamination or sequence analysis issues.

Pyrosequencing was performed on 30 individual parasites per population whilst deep amplicon sequencing was run using pools of 500 – 1000. Given the greater number of individuals present in the template and the depth of sequencing afforded by this technology; typically 10,000 reads per sample, detection of low frequency alleles would be expected using deep amplicon sequencing, as illustrated by previous works (Nilyanimit et al., 2018). The use of 30 individuals in pyrosequencing analysis balanced the resources available with the depth of data required to reliably estimate the resistance status of each population and followed the method used in previous studies (Redman et al., 2015; Skuce et al., 2010). Limiting the number of individuals per population restricts the power of the analysis; sampling errors could occur, meaning very low resistant allele frequencies could be missed or, conversely, that resistance could be over-represented. Due to the multiplication factor, sensitivity of the pyrosequencing assay was 3%, thus resistant allele frequencies >10% should be reliably detected. Despite different numbers of parasites being included in the templates for each method, the outputs were compared in this study to assess whether the high-throughput MiSeq technique provided comparable results to the currently used pyrosequencing method.

DNA extracts were prepared for pyrosequencing analysis shortly after sample collection however, those for deep amplicon sequencing analysis were prepared at a later date from EtOH-fixed parasite material. Differences in allele frequency may therefore represent preferential die-off of individuals carrying the resistant allele prior to EtOH fixing. Although it has been hypothesised that the F200Y SNP does not carry a fitness cost in other GIN species (Elard et al., 1998), viability may be reduced in *N. battus* at low allele frequency. Life stage-specific differences in DNA abundance may also have influenced the allele frequency however, attempts were made to analyse a similar mix of life stages with both methods to minimise this impact.

Variation in results may have arisen through sequencing or amplification errors in either method. A small degree of sequencing error is unavoidable, regardless of the platform used. The error rate associated with deep amplicon sequencing has been found to vary with the rate of clusters passing the filter but is estimated at around 0.1% (Glenn, 2011). Pyrosequencing error has been estimated at around 5% (Barrere et al., 2012) however, additional errors will likely be introduced at a low rate during pre-sequencing PCR in both methods, so may be higher than these figures. Contamination is also a possible cause of variation however, appropriate measures were taken to ensure that the risk of contamination was minimised at each stage of sample preparation and analysis.

Bias was identified in previous deep amplicon sequencing studies of nemabiome analysis (Avramenko et al., 2015). Despite the mono-species analysis conducted in the present study, bias between susceptible and resistant haplotypes may be possible. The binding sites of primers were conserved regions and thus, it is unlikely that differences in binding site sequence were present however, amplification efficacy may have been different due to sequence variation between resistant and susceptible haplotypes. Samples run in triplicate during the current study produced comparable results, if bias exists in the amplification efficacy between haplotypes then this was consistent between replicates, as found in the nemabiome analysis. Bias within the nemabiome assay was accounted for using correction factors, calculated following detailed analysis of mock samples. A similar validation using mixtures of pre-tested lysates could be designed for the current study. Further research would be required to assess all possible routes of bias in this assay.

Despite variation in results between the two methods at low allele frequency, we conclude that either method would be suitable as a diagnostic tool. The frequency of F200Y and F167Y were found to be low in the majority of populations tested at present, therefore pyrosequencing may provide a more reliable diagnosis of low allele frequencies. However,

research in other nematode species would suggest that resistant allele frequencies below 10% are unlikely to cause clinical drug inefficacy (Cudekova et al., 2010; von Samson-Himmelstjerna et al., 2009) therefore deep amplicon sequencing would provide sufficient resolution as a tool to diagnose possible anthelmintic resistance following suspected drug failure. Deep amplicon sequencing is capable of analysing a large number of pooled samples in a single run using unique barcoded primer pairs, this high throughput method is therefore suited to large-scale projects or central diagnostic laboratories. Pyrosequencing analysis has been conducted using pooled samples (Esteban-Ballesteros et al., 2017) however, the results obtained were found to be less reliable than individual analysis during validation steps (data not shown). Sequencing from individuals is labour-intensive and expensive on a large scale however, provides detailed information on individual allele frequencies. As a research tool, information on the ratio of hetero- and homozygous genotypes provides valuable insight into the stability of resistance and can be used to estimate whether a genetic locus is currently under active selection by comparison to the Hardy-Weinberg equation (chapter 2). The analysis of individual parasites by pyrosequencing may be a more suitable and cost-effective method for small-scale applications.

An additional benefit of deep amplicon sequencing is the wealth of data produced which, given the development of an appropriate analysis pipeline, could provide information on haplotype diversity, phylogenetics and population structure. The additional information could be used to investigate the potential origin(s) of benzimidazole resistance in *N. battus.* Three main hypotheses exist regarding the likely origin(s) of benzimidazole resistance-associated SNPs; pre-adaptive mutations existed in the population prior to the onset of selection pressure, de novo mutations which each arose by chance and disseminated from single sources or recurrent mutations, disseminated from multiple locations (Gilleard and Beech, 2007). *N. battus* provides a unique opportunity to study the origins of resistance due to the recent emergence of BZ-resistance in this species, low frequency mutations and

restriction of the species to temperate regions. As discussed in chapter 1, studies on *H. contortus* and *T. circumcincta* reported evidence that F200Y likely arose through recurrent mutations in multiple locations but suggested that E198Y and F167Y may have disseminated from rare mutations at a single/few source(s) (Redman et al., 2015; Silvestre and Humbert, 2002; Skuce et al., 2010). Differences were observed between these species in resistant haplotype diversity, believed to result from different life history traits of the two species (Silvestre and Humbert, 2002). The development and dissemination of BZ-resistant SNPs in *N. battus* may therefore be different again as this species has a unique epidemiology compared to other GIN.

This study was the first identification of the F167Y SNP in *N. battus.* Primary characterisation of the initial BZ-resistant isolate by Morrison *et al.* (2014) identified F200Y as the major SNP conferring resistance in this species. Due to the narrow target locus of pyrosequencing (~50bp), the 167 locus was out-with the possible range when analysing the F200Y locus and as a result, additional differences were missed during initial testing. Deep amplicon sequencing sequences a larger target region; typically ~450bp, allowing for the identification of F167Y during the current study. Unlike MiSeq, pyrosequencing requires information on the expected sequence of nucleotides surrounding the SNP of interest prior to analysis and so is unable to detect unknown changes. Following the development of a pyrosequencing assay for F167Y, it was successfully identified and quantified using both pyrosequencing and deep amplicon sequencing technologies. Differences between the platforms were expected for F167Y given the low allele frequency (max 13%). As previously described, DNA samples comprised different individual parasites, possibly introducing sampling errors and the low prevalence is at the limit of the 3% sensitivity rate of pyrosequencing given the number of individuals sequenced. Identification of the resistant allele in two populations by deep amplicon sequencing where it was not detected by pyrosequencing was likely due to the

larger number of individuals included in the template DNA, increasing the likelihood of rare resistant alleles being present.

4.6 Conclusions

Deep amplicon sequencing and pyrosequencing assays have been developed for the detection and quantification of SNPs associated with benzimidazole resistance in *N. battus*. Results from the two platforms were comparable for the F200Y mutation which is believed to be key in conferring BZ-resistance in the species. Results from position 167 analysis were not comparable however, given the low frequency, this was as expected. Both diagnostic methods described could be utilised to monitor the development and dissemination of SNPs in *N. battus* and confirm BZ-resistance in cases of suspected clinical drug failure. The reduction in labour costs when analysing pooled samples is a major benefit of deep amplicon sequencing over pyrosequencing. As a diagnostic tool, the number of populations which can be analysed in a single run makes deep amplicon sequencing a more economic option compared to pyrosequencing given a large sample bank however, detailed information from sequencing of individual eggs/L3 is lost by this method. Pyrosequencing provides a fast, detailed quantification of SNPs from individuals, suited to analysis of small sample sets. Deep amplicon sequencing provides vast amounts of data over a greater area of the gene, allowing for detection of unknown differences as well as quantification of known SNPs. Detection of the F167Y SNP during F200Y quantification was of great benefit to the current study. The MiSeq platform is capable of producing extensive data sets which, given the development of appropriate bioinformatics pipelines could be utilised as powerful tools both in research and diagnosis of anthelmintic resistance for veterinary and medical diseases. The use of either method for the detection and quantification of BZ-related SNPs would be useful in monitoring the development of BZ-resistance in *N. battus* over time and potentially informing anthelmintic choice. Accurate detection of BZ-resistant alleles, together with

knowledge on the drivers associated with resistance could be used in the future to inform control strategies aimed at slowing the development and dissemination of resistance in this species.

5 Descriptive analysis of UK farm management practices and current *N. battus* control strategies

5.1 Abstract

Farm management and parasite control strategies have a major impact on the parasite population on farm, from population dynamics and contamination levels to the rate of development of anthelmintic resistance. Understanding the current range of management strategies in use throughout the UK to control *N. battus* is required to assess the impact that farm management decisions have on the changing epidemiology and emerging resistance observed in this species. Questionnaires were developed and distributed to the farming community via a range of dissemination channels including direct email, social media and with help from veterinary practices and agricultural bodies promoting the survey. The survey consisted of 42 questions based on grazing and grassland management, farm demographics and details of parasite control strategies including, anthelmintic usage and monitoring methods. A total of 197 completed questionnaires were collected. Descriptive analysis was conducted to explore regional variation in the timing and severity of infection and control measures employed on farms. Respondents' perception of *N. battus* disease severity varied regionally with more severe disease being reported in the North. Farms in the South observed greater changes in the timing of disease with *N. battus* being reported throughout the year on some farms. Farms in Scotland and North England typically indicated that *N. battus* infection was restricted to spring and summer. Evidence based anthelmintic usage was greatest in the South with a statistically significantly higher use of faecal egg counting and online risk maps. Geographic trends in *N. battus* and epidemiological changes may reflect climatic or management differences throughout the country influencing the parasite population dynamics. Information collected during this study on farmer perceptions of *N.*

battus infection and the control practices currently being used for this species will help inform risk factor analysis and the development of future sustainable control strategies.

5.2 Introduction

Nematodirus battus is an economically important parasite of sheep which threatens lamb health and the sustainability of UK sheep farming sustainability in the UK. The use of benzimidazoles and grazing strategies which avoid repeated grazing of lambs on the same pasture each spring have both been advocated for the control of *N. battus* in sheep. However, the extent to which these practices are employed and their effectiveness is unknown. Management strategies have a significant impact on the parasite population, beyond within-year control, influencing refugia level, selection pressure and the availability of suitable hosts which in turn will impact parasite adaptation. With apparent changes in epidemiology of *N. battus* and the emergence of BZ-resistance in this species, it is important to understand current management throughout the UK and the rationale behind these control strategies to support the development of optimised control practices in the future.

The current study aimed to explore farmers' perceptions of *N. battus* in their farming systems and how this parasite has changed epidemiologically and pathogenically in recent years whilst gathering information on farm management strategies in use throughout the UK. The questionnaire analysis will provide detailed information about current management and control measures being used and how these vary between farms. Observing various changes in this parasite is essential to inform research into the impact of management practices on parasite biology and population dynamics. The knowledge gained will be valuable in the development of effective control strategies to avoid production costs and protect lamb welfare.

We hypothesise that *N. battus* disease severity and management practices will vary geographically throughout the UK. We will explore the uptake of currently advocated control

strategies, including varied grazing of lambs in spring, treatment of young lambs with benzimidazoles and disease monitoring methods, which are believed to reduce the risk of *Nematodirosis* in lambs using an online questionnaire distributed to study farms and the wider farming community.

5.3 Materials and Methods

5.3.1 Interviews

Interviews were conducted on seven study farms to inform question development. Farms were selected based on the resistant allele (F200Y) frequency observed in the genotyping survey being greater than 10% (Chapter 2). The interviews were semi-structured, i.e. free conversation guided by a series of broad questions, conducted in an informal setting on each farm (see appendix 3 for interview structure questions). Six of the interviews were un-mediated; involving the farmer and two researchers, the final interview was also attended by two local vets. Each interview lasted approximately one hour and all sessions were recorded. All participants were briefed and signed a consent form prior to the interview.

5.3.2 Questionnaire design

A 42-question questionnaire was developed. It was divided into four sections; i) farm demographics, ii) farmer perceptions of *N. battus* and specific control practices, iii) grazing management and iv) general anthelmintic usage and quarantine. The questionnaire was developed, disseminated and responses collected and collated using the *SurveyMonkey* platform. See appendix 4 for a copy of the full questionnaire.

Questions were designed to gather information on respondent's perceptions of *N. battus* on their farm, the severity of disease, in which season signs of *N. battus* were typically observed and their perception of if/how the disease had changed within the five years prior to the survey. General management practices focused on husbandry decisions which may interfere

with or promote the lifecycle of *N. battus* and control strategies aimed at *N. battus*, other GIN species and *Fasciola hepatica* throughout the grazing season.

5.3.3 Question selection

A total of 135 questions were written during questionnaire development, reduced to 42 questions in the final questionnaire. The survey length was designed to balance the depth of information collected with minimising the length of time required for completion, thus encouraging farmers to take part. Questions were selected to provide the farm management information required to conduct risk factor analysis into the development of BZ-resistance (chapter 6) and varied hatching behaviour of *N. battus* (chapter 7).

Questions referring to calibration of equipment and technical aspects of anthelmintic administration were not included in the current questionnaire as the association of these factors with anthelmintic resistance has been addressed by previous authors (McMahon et al., 2017). Details of anthelmintic treatments administered to control strongyle nematodes were retained however, questions relating to anthelmintic resistance in strongyle species were removed as resistance in other nematode species would not necessarily constitute a risk factor for resistance development in *N. battus*. Social science questions were also kept to a minimum, respondents were asked about their experience of *N. battus* infection on farm however, questions relating to the motivation for anthelmintic choices and drug class rotation were removed. Questions asking for sensitive information about productivity and growth rates and those relating to animal movements, markets used and trade relationships were removed. The ease of analysis was also considered therefore questions relating to specific fields (e.g. gradient, orientation, UV, drainage) or groups of animals (e.g. within farm movements and mixing of groups) where multiple answers may be given for different fields on large farms, were removed.

5.3.4 Piloting

The online questionnaire was pilot tested by four volunteer farmers. Pilot testers reported any issues encountered or unclear language choices and recorded the length of time spent completing the questionnaire.

5.3.5 Dissemination

Farmers submitting samples for inclusion in the genotyping survey (chapter 2) were contacted by direct email including a link to the online questionnaire. One further email remainder was sent and study farmers who did not complete the online survey were followed up by post to eliminate loss of involvement due to incorrect contact details.

The link to the questionnaire was disseminated to the wider farming community through social media, veterinary newsletters and publication of short articles. The link was shared on twitter by several agricultural groups including the Scottish farmer, members of the AHDB research team and veterinary practices. A short article about the project including a link to the questionnaire was included in the national sheep association (NSA) weekly email newsletter and several veterinary practices included the link on newsletters and posted about the survey on social media. Moredun foundation regional advisors also circulated the link to their local farming community (Appendix 5).

5.3.6 Analysis

Questionnaire responses were downloaded from *SurveyMonkey* into Microsoft Excel for data cleaning, coding and analysis. Descriptive analysis was conducted for each question, initially using the full data set then split by regional location (Figure 2.2). Chi-squared analysis was conducted in R (version 3.2.5) to explore significant geographical differences in in management factor application frequency. Where factors were compared between North and South, responses from North Scotland, South Scotland, North East and North West

England comprised “the North” and South East, South West, South central England and Wales comprised “the South”.

Faecal egg count data from the Animal and Plant Health Agency (APHA) parasite surveillance centres in England and Wales was collated from January 2015 to December 2017. The number of diagnostic submissions positive for *N. battus* in the North (North East and North West), South (South East, South West, East Anglia and the midlands and South Central) and Wales and the mean number of *N. battus* eggs per gram was calculated for each month of the year. Analysis of the APHA data was carried out using Microsoft excel. A statistical significance threshold of 5% (p=0.05) was used in all analyses.

5.4 Results

5.4.1 Farm demographics

A total of 197 completed questionnaires were collected; 87 farms involved in the project who provided samples for genotypic analysis (chapter 2) and a further 110 responses from the wider farming community. Responses were collected from farmers throughout the UK (Figure 5.1). Regions were divided based on postcode (see Figure 2.2); 9 responses from North Scotland, 13 from South Scotland, 39 from North East England, 17 from North West England, 23 from South central England, 32 from South East England, 41 from South West England and 13 from Wales. Appendix 6 contains a summary of the questionnaire results overall and by region, including the number of responses per question.

Farms involved in the questionnaire study were largely commercial flocks (84%) who bred and finished lambs on farm (64%). A higher proportion of the respondents from Wales managed pedigree flocks (33%) compared to the UK as a whole (16%) and this was also reflected in the number of breeders in this region; 33% of Welsh respondents were solely breeders compared with 25% of the respondents overall. The majority of respondents had

been farming their current land for more than 20 years (51%); 20% for less than five years, regional differences were marginal.

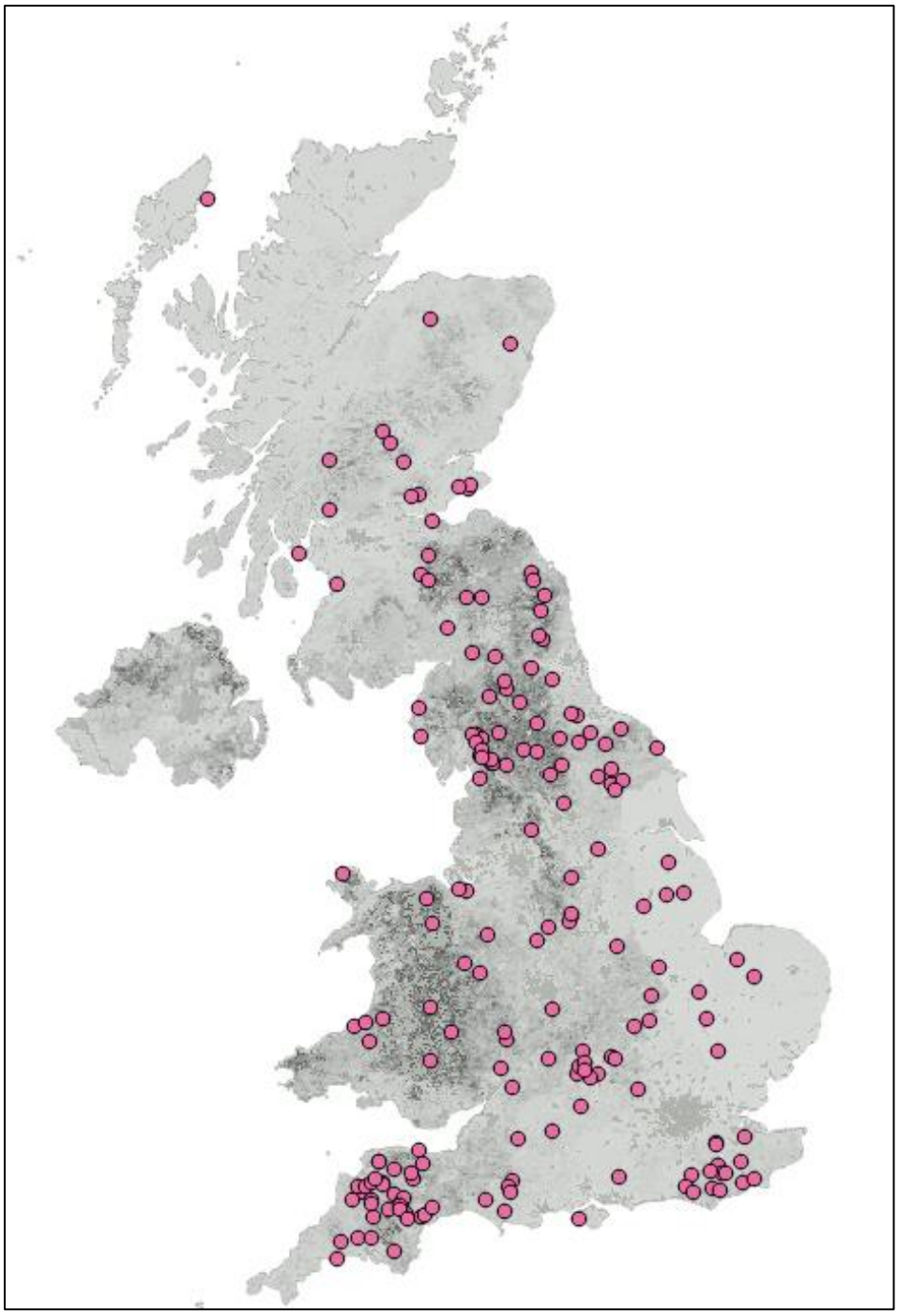


Figure 5.1. Map of questionnaire respondents.

UK Map overlaid with sheep densities (sheep density data from the Office for National Statistics in 2009 was mapped using QGIS (Las Palmas version 2.18); data source Geo-wiki) showing the location of questionnaire respondents in relation to sheep-dense locations.

Farms included in the study varied in size, the median number of ewes kept was 250 (80, 693) (median (Q1, Q3)) (range 20-5000), 9 (3, 17) rams (range 0-175) and 347 (118, 915) lambs (range 20-7000). The UK livestock census reported the national average to be 230 breading ewes in 2016 (Department for Environment, 2017), indicating that the farms included in this study were representative of UK sheep farming in terms of flock size. The largest farm included in our survey was located in South East England, running 5000 ewes and 7000 lambs however, southern Scotland had the highest number of ewes on average per farm; median 960 (350, 1100). 57% of respondents managed mixed cattle and sheep enterprises and 3% farmed goats and sheep. Median number of goats kept was 30 (7, 40), range 1-200 and the median number of cattle kept was 80 (20, 160) (range 2-940).

Lambing was most common in March and April and weaning peaked between July and August on surveyed farms. Peak lambing dates did not appear to vary between regions (Figure 5.2).

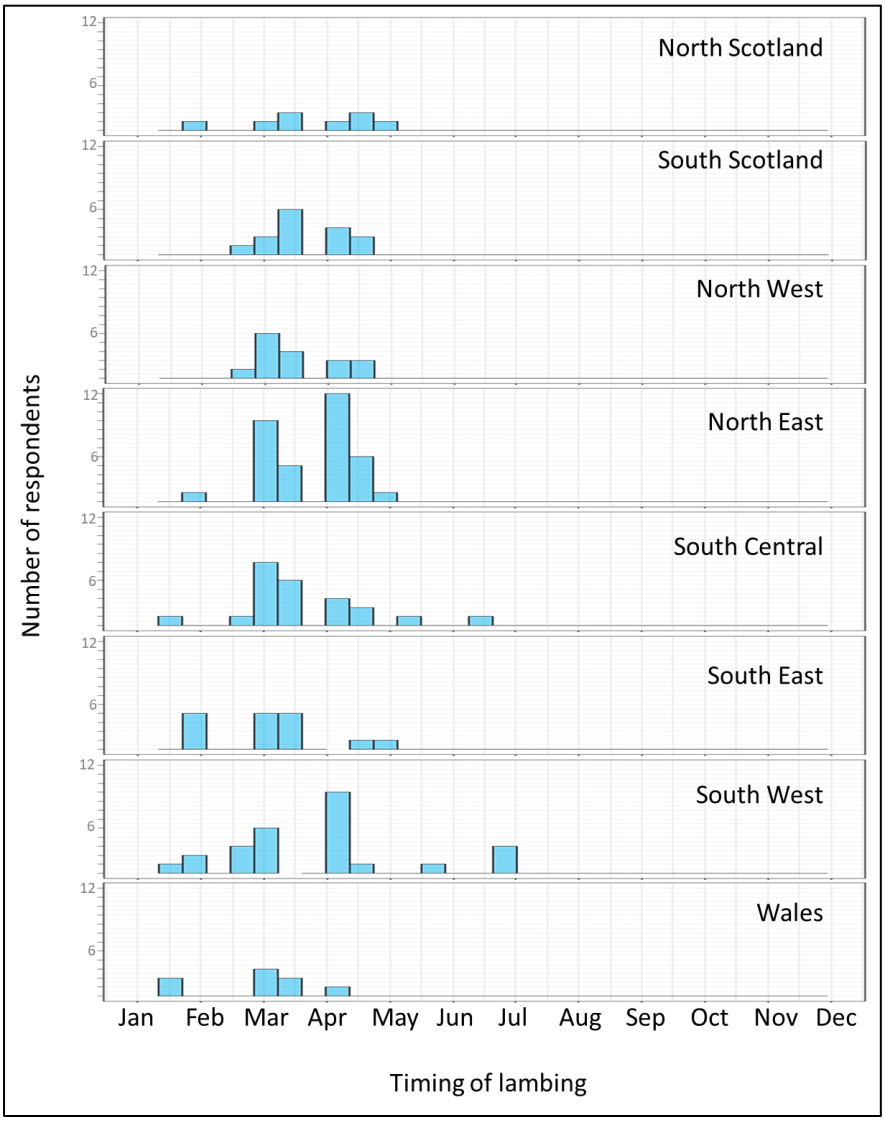


Figure 5.2. Histogram plots of the timing of peak lambing on farms split by geographic region.

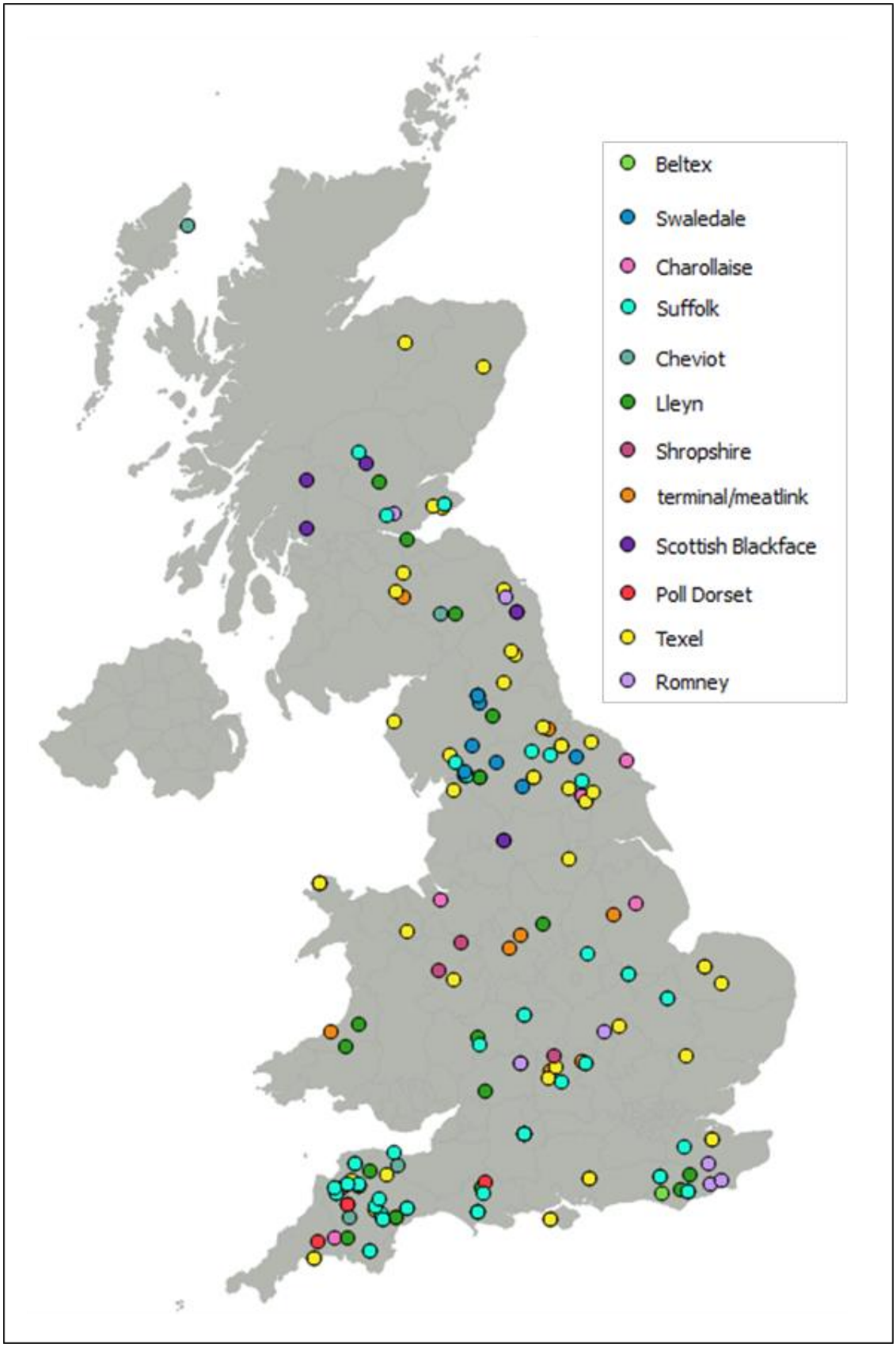


Figure 5.3. Map of main sheep breed kept by questionnaire respondents.

Respondents kept a range of sheep breeds (Figure 5.3). Texel, Suffolk and Lleyn breeds were reported throughout the UK however, others were found to be more regionally aggregated such as, Blackface sheep in Scotland, Swaledale in the North of England and Poll Dorset in the South.

5.4.2 Perception of N. battus on farm

Respondents answering 'yes' to the question "have you had *Nematodirus* on your farm in the last five years?", were asked to rate the severity of disease observed; choosing from severe signs (scouring and deaths), low level infection (scouring but no deaths) or sub-clinical (present but no clinical signs of infection). The majority of farms surveyed (76%) had diagnosed or experienced clinical signs of *N. battus* within the past five years, with 56% observing scouring in lambs. Annual lamb losses occurred in 14% of flocks. Although perceived to be present, *N. battus* was not formally diagnosed on 39% of farms surveyed.

Comparison of *N. battus* symptom severity ratings between respondents in Scotland and North England to those from South England and Wales identified significant variation from North to South (χ^2= 9.1, d.f.= 3, p= 0.03). Respondents reported more severe signs of disease in the North compared to the South; almost 20% of respondents in the North experienced severe scouring and lamb losses each year compared to around 10% in the South. The opposite was true of sub-clinical infection; 28% of respondents in the South observed subclinical *N. battus* infection each year compared to 10% in the North. No significant difference was identified when comparing *N. battus* symptom severity scores between moderate and large farms (up to 500 ewes and over 500 ewes) or between mixed stock farms and sheep only enterprises by Chi-square analysis (χ^2= 2.8, d.f.= 3, p= 0.4 and χ^2= 0.6, d.f.= 3, p= 0.9 respectively).

5.4.3 Timing of infection

Respondents were asked which season(s) they typically observed signs of *N. battus* on farm. Characteristic scouring and lamb losses were observed most commonly in spring however, signs were identified throughout the year in different systems. Spring-only transmission (March–May) was observed by 51% of respondents, 21% reported observing signs exclusively in summer months (June–August) and 3% in autumn only (September–November). Figure 5.4a shows the overall proportion of respondents who observed signs of *N. battus* in each season.

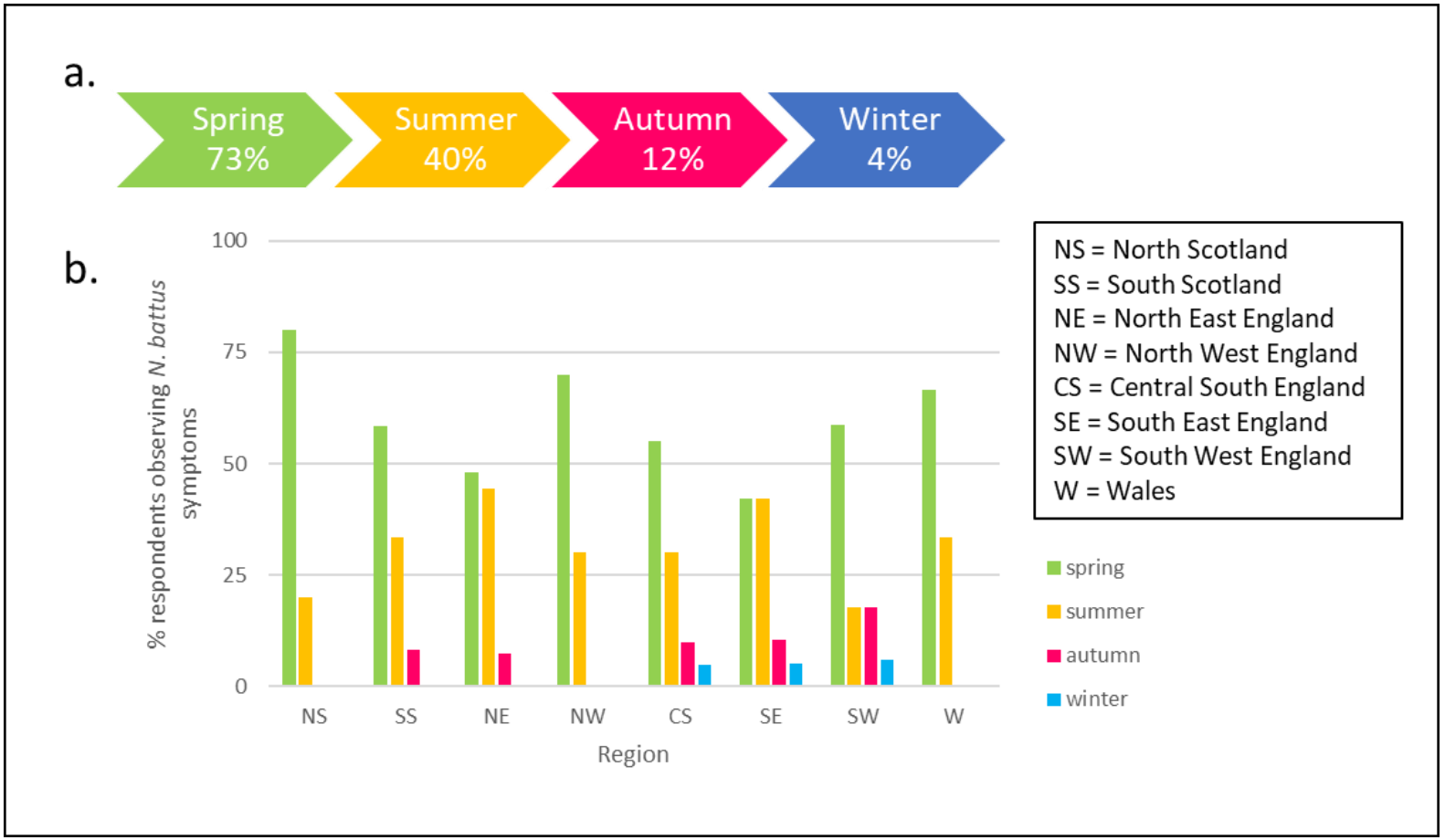


Figure 5.4. Summary of the season in which respondents typically observed symptoms of N. battus.

The proportion of respondents observing typical signs of N. battus during each season (a) overall and (b) by region. Green bar (spring); Yellow bar (summer), Red bar (autumn); Blue bar (winter).

The timing of observed signs of *N. battus* varied regionally (Figure 5.4b). Respondents from the North of Scotland predominantly observed *N. battus* in spring, 20% also reported infection in summer but no autumn disease was reported in the region. Respondents from North West England and Wales reported similar disease timing as North Scotland. In contrast, symptoms of *N. battus* infection were reported throughout the year in the south of England. Typical spring infection accounted for only 44% of observations in South East England, 6% of respondents in this region observed signs of infection in autumn and a further 6% in winter. A small proportion of respondents from southern Scotland and North East England also reported autumn *N. battus* on farm (8% and 7% respectively). Despite more reports of disease incidence in autumn and winter in the south compared to the north, no statistically significant difference was identified (χ^2= 2.3, d.f. = 1, p= 0.1). The observation of symptoms in different seasons did not appear to be associated with lambing date which was similar between regions (Figure 5.2).

Parasite surveillance data collated from diagnostic samples submitted to Animal and Plant Health Agency (APHA) from farms throughout England and Wales, followed a similar pattern to the farmer observations from the current study (Figure 5.5). The APHA data illustrated an autumn/winter peak in the number of submissions positive for *N. battus* in the South which was not apparent in the North. The mean faecal egg count was lower in autumn samples compared to those submitted during the spring/summer peak however, still represents significant contamination of pasture late in the season.

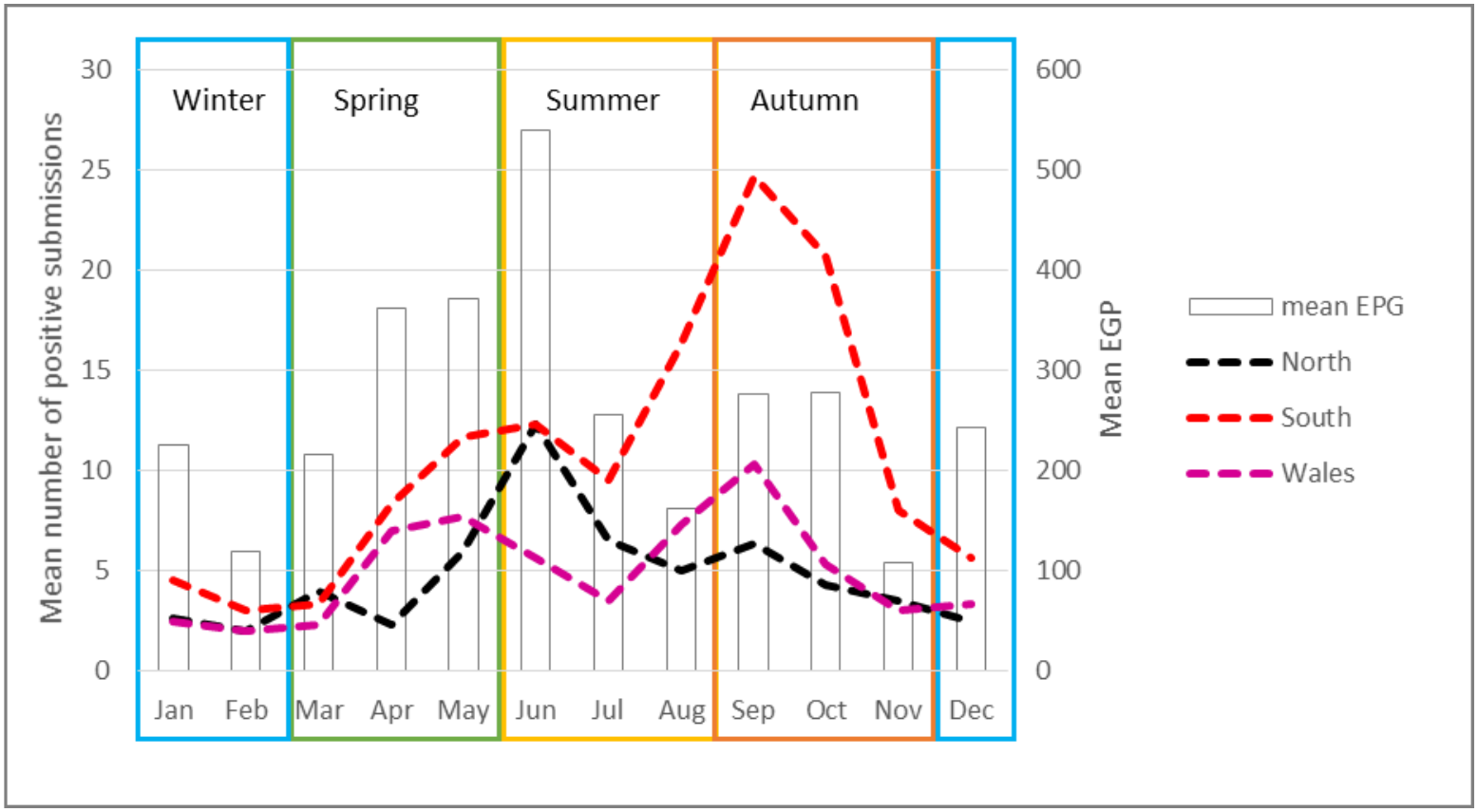


Figure 5.5. Animal and Plant Health Agency N. battus surveillance data for England January 2015/December 2017.

Comparison of the number of diagnostic submissions found to contain N. battus throughout the year in the North (North East and North West in red), Wales (purple) and south (South East, South West, East Anglia and the midlands and South Central in black). The bars represent the mean N. battus faecal egg count of diagnostic samples submitted per month.

The timing of observed signs of *N. battus* infection also varied by farm type. Respondents from hill farms reported observing infection more commonly in summer than spring (41% and 35% respectively) but autumn infection was reported by a similar proportion of respondents from each farm type; 7%, 8% and 6% in lowland, upland and hill farms respectively (χ^2= 0.05, d.f.= 2, p= 0.98). The small number of winter incidences were exclusively reported on lowland farms.

5.4.4 Changes in *N. battus* infection on farm in recent years

Respondents were asked how *N. battus* infection had changed temporally within the five years prior to the study. The type of changes respondents perceived on farm (shift in the timing and/or severity of *N. battus* infection) were compared between farms in the North and South of the UK and were found to be statistically significantly different between regions (χ^2= 16.38, d.f. = 5, p= 0.006) (Figure 5.6).

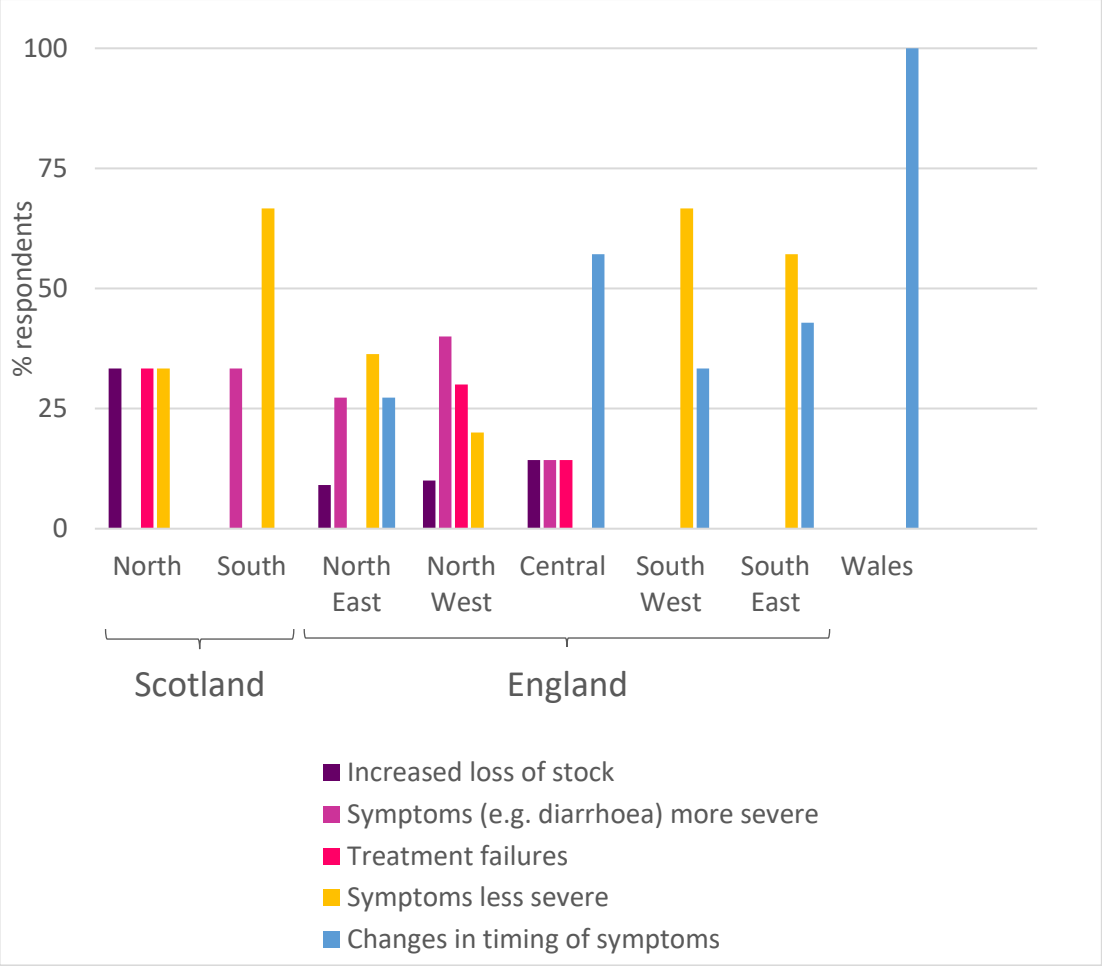


Figure 5.6. Summary of the changes in N. battus infection reported by respondents.

Proportion of respondents in each geographic region observing different changes in N. battus disease on farm (timing/severity of symptoms and outcome of anthelmintic treatment) within the five years prior to the study.

Respondents from the South of England reported a shift in the timing of *N. battus* infection on farm from typical spring/early summer infection to autumn/winter disease incidence (Figure 5.6). Respondents from Wales also reported changes in the timing of *N. battus*, however, these respondents still considered spring/summer to be the main peak of *N. battus* on their farm. APHA surveillance data showed a peak in the number of *N. battus* positive submissions from Wales around September suggesting that Autumn *N. battus* does occur in this region however, it does not present as significant a threat as spring disease.

Respondent observations of changes in the severity of the signs of *N. battus* infection within the five years prior to the survey varied North to South (Figure 5.6). Increased severity of disease, treatment failure or increased loss of stock was reported by 63% of respondents from Scotland and North England, compared to 27% from the South however, the geographical difference was not found to be statistically significant (χ^2= 2.5, d.f.= 1, p= 0.1).

5.4.5 Grazing management

The majority of respondents (88%) repeatedly grazed young lambs on the same fields each spring, creating fields which represent a 'high risk' for *N. battus* transmission. When asked about grassland management of 'high risk' pasture, almost all respondents (98%) also grazed this land in autumn, most commonly with ewes (60%). Few respondents reported having reseeded 'high-risk' pastures within the previous five years (22%).

The majority of respondents (51%) employed rotational grazing or frequently moved lambs around the farm whilst 38% of respondents set stocked lambs. Grazing strategy was found to be statistically significantly associated with the length of time respondents had been earning a living from farming at their current address; a proxy for age and farming experience (χ^2= 15.5, d.f. = 6, p= 0.02). Set stocking was most common amongst those farming their current land for more than 20 years (45%) compared to only 25% of those farming for less than five years. Respondents from Scotland and North West England employed set stocking more often than southern regions (63% and 28% respectively) (χ^2= 9.59, d.f. = 3, p= 0.02).

Complex, labour intensive grazing practices such as cellular and leader/follower systems, where the most productive animals graze first and less productive stock follow through the paddocks, were less commonly practiced compared with set stocking and rotational systems (6%, 5%, 38% and 51% of respondents respectively). These methods were reported throughout England but not in Scotland or Wales with the exception of one respondent.

Stocking density pre- and post-weaning varied considerably between farms (range 0.8-47 and 0.8-35 heads per acre, pre- and post-weaning). However, no association was observed between stocking density and region (χ^2= 0.2, d.f. = 7, p= 1).

Respondents were asked about co-grazing of lambs with other livestock species and grazing a proportion of animals on land separate from the main farm each year (away grazing). Around half of respondents (51%) co-grazed lambs with other livestock. Of those employing mixed species grazing, 74% did so on a sporadic basis, 9% post-weaning and 17% co-grazed permanently. The majority of co-grazing was with beef cattle, 57% with young beef cattle and 52% with adult cattle.

Away grazing was reported by almost half of respondents, most commonly in the north of England with 41% of respondents in the east and 21% of respondents in the west routinely grazing a proportion of lambs off farm each year. A further 16% and 21% of respondents from the east and west respectively sporadically 'away grazed' lambs. Mixed species grazing and away grazing were not found to vary significantly between regions (χ^2= 4.6, d.f. = 7, p= 0.71 and χ^2= 5.6, d.f.= 7, p= 0.59 respectively).

5.4.6 Anthelmintic control

5.4.6.1 N. battus

BZ compounds remain the most common choice of anthelmintic for the control of *N. battus*; 87% of respondents administered BZ compounds to control *N. battus* within the 12 months prior to the survey (Table 1). Despite the continued use of BZ compounds to control *N. battus*, the number of respondents relying solely on this anthelmintic class appeared to decrease within the five years prior to the study as the proportion of respondents using multiple anthelmintic classes to control *N. battus* increased from 17% to 54%. The reduction in sole BZ usage was coupled with an increase in the use of levamisole (LV) and macrocyclic

lactones (ML) whilst administration of the two novel anthelmintic compounds monepanel (AD) and derquantel/abamectin (SI) remained low for *N. battus* control.

5.4.6.2 GIN

The most common anthelmintic class used to control other GIN species was the ML (67% of respondents) followed by BZ (52%). Over half of the respondents (58%) used multiple anthelmintic classes in the five years prior to the survey (see Table 5.1 for a breakdown of anthelmintic usage). The results show a noticeable uptake of AD but usage of the other new active SI remained low; 20% and 6% respectively.

Table 5.1. Summary of anthelmintic classes used to control N. battus and other nematode species.

Percentage of respondents administering each anthelmintic class to control N. battus in the year of the study (Nb 1 yr) and within the five years prior to the study (Nb 5 yr) and other GIN species within the 5 years prior to the study (GIN) and the proportion of respondents administering single or multiple anthelmintic classes.

	% 1-BZ	% 2-LV	% 3-ML	% 4-AD	% 5-SI	**% single class**	**% multiple classes**
Nb 1 yr	87	21	29	5	1	**46**	**54**
Nb 5 yr	84	10	18	2	2	**83**	**17**
GIN	52	42	67	20	6	**42**	**58**

On average, lambs received three treatments per year (mean±SEM, 2.8±0.11), ewes and rams typically received two treatments per year (1.9±0.09 and 2.0±0.09 respectively). The average number of anthelmintic treatments administered to ewes was higher in the North of England and Scotland compared to the south of England (mean±SEM) 2.4±0.4 drenches per year compared with 1.5±0.2. However, variation in the number of anthelmintic treatments administered to ewes, rams and lambs each year was not statistically significantly different between regions (χ^2= 0.51, d.f. = 14, p= 1).

Respondents were asked which method they used to determine when to administer anthelmintic treatments to control *N. battus* and GIN in lambs and adult sheep, results are summarised in Table 5.2.

Table 5.2. Summary of parasite monitoring methods used to determine the timing of anthelmintic treatment.

Percentage of respondents using each method for monitoring parasitic infection to determine the optimal time to administer anthelmintic treatment to lambs, ewes or rams to control N. battus and other GIN species.

	N. battus	Roundworms (Trichostrongylid spp. other than *N. battus)*			Overall (all worm control – *N. battus* and Trichostrongylid spp.)
	Lamb	Lamb	Ewe	Ram	
Faecal egg count	41	56	28	21	63
Ill thrift	46	42	18	15	59
Pre/post- lambing	8	5	57	22	59
Mating	4	5	28	42	47
Set time	17	18	17	20	34
Risk map	31	7	3	2	33
Weight gain	11	17	3	1	19
Veterinary plan	12	12	15	13	20

Overall, faecal egg count monitoring was selected as the most common method of determining when to administer anthelmintic treatment to sheep throughout the grazing season to control parasitic infection, utilised by 63% of respondents.

Treatment timing to control *N. battus* infection in lambs was most commonly determined using ill thrift and faecal egg counting (46% and 41% of respondents respectively). A third of respondents reported using online risk maps to predict the optimal time for treatment. Uptake of risk maps appeared to vary regionally; used by 17% of respondents from Scotland

and northern England compared to 31% from southern England and Wales but the difference was not statistically significant (χ^2= 0.7, d.f.= 1, p= 0.392).

The timing of anthelmintic treatments to control other trichostrongylid species were most commonly determined by faecal egg count monitoring for lambs (56% of respondents) however, less than half of those respondents also used FEC in adult sheep (28% in ewes and 21% in rams). Other evidence-based drenching methods such as live weight monitoring were less common; used by 20% of respondents, largely for the control of GIN in lambs. Ill thrift was the second most common diagnostic, used by 59% of respondents to determine the timing of anthelmintic treatment. Despite a high proportion of the respondents indicating that they used ill thrift monitoring, only two respondents in the current study relied solely on the observation of ill thrift to determine when to administer treatments.

Around 34% of respondents administered anthelmintic treatment at the same time each year and a further 20% utilised veterinary treatment plans, which typically include FEC monitoring and the use of risk maps. Yearly, prophylactic drenching was similar for lambs and adult sheep, as was reliance on veterinary plans. Anthelmintic usage in adult sheep was linked with the timing of other farming events for which these animals would be gathered and handled i.e. mating and lambing, on the majority of farms surveyed.

Monitoring methods varied regionally for GIN control with evidence-based treatment decisions (FEC/weight gain/risk map/vet plan) more commonly reported in the South of England than the North and Scotland (31% and 45% respectively).

Table 5.3 summarises the use of FEC and yearly prophylactic treatments between regions. Variation in the use of FEC monitoring was statistically significant between regions (χ^2= 19.7, d.f. = 7, p= 0.006) most commonly employed in south west and South Central England (80% and 81% of respondents respectively). The use of prophylactic, yearly treatments also

appeared to vary North to South with 40% and 39% of respondents in South Scotland and North East England employing yearly treatments respectively, compared to 22% and 20% of respondents in Wales and South West England respectively however, regional variation was not found to be statistically significant (χ^2= 7.4, d.f.= 7, p= 0.4).

Table 5.3. Regional use of faecal egg counting and yearly prophylactic treatments in the control of GIN. Percentage of respondents from each region employing faecal egg counting to determine the optimal timing of anthelmintic treatment compared to the percentage using yearly, prophylactic anthelmintic treatments to control GIN infection in grazing sheep.

Region	Faecal egg counting	Prophylactic yearly treatment
North Scotland	17%	50%
South Scotland	54%	46%
North East England	52%	39%
North West England	27%	64%
South central England	81%	24%
South East England	44%	30%
South West England	80%	20%
Wales	67%	22%

Monitoring methods were largely similar between lowland and upland farms however, hill farms were found to use more pre-planned annual treatments; 64% compared to 30% of lowland and 33% of upland farms (χ^2= 6.3, d.f.= 2, p= 0.04).

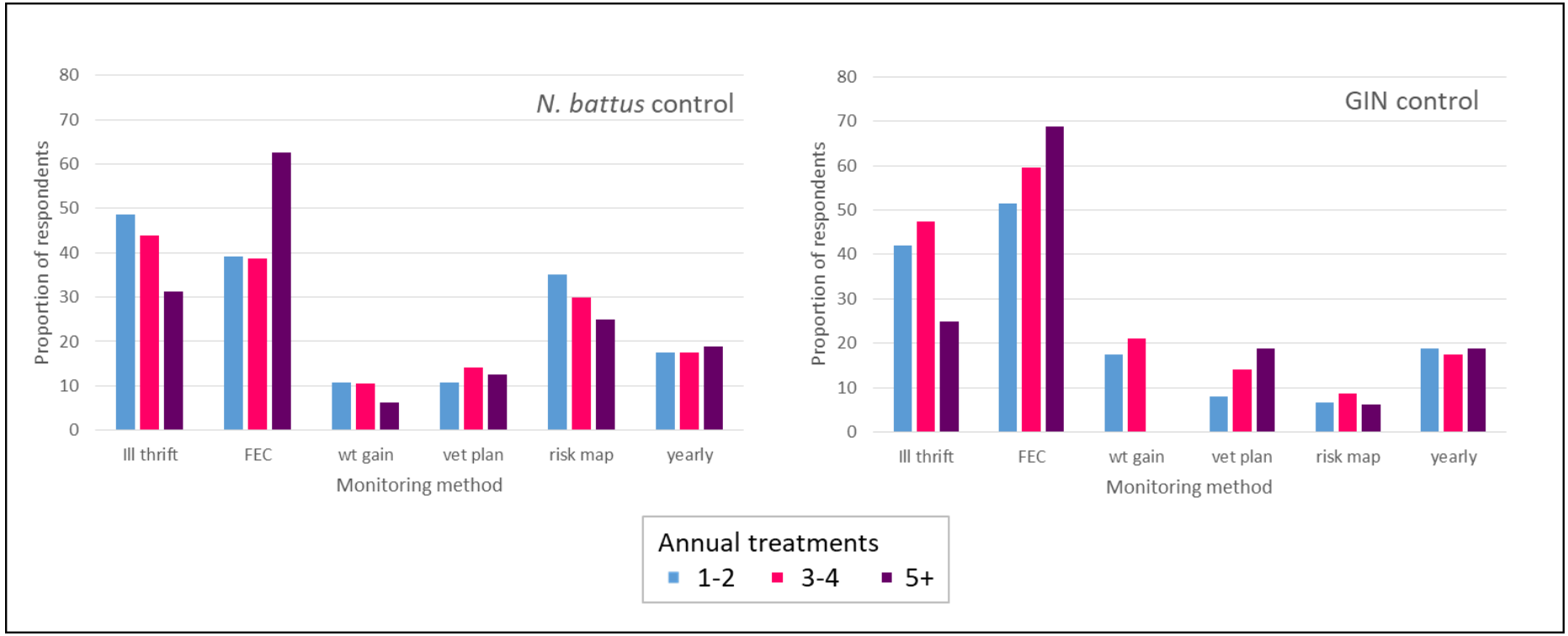


Figure 5.7. Comparison of parasite monitoring method and the typical number of anthelmintic treatments administered annually to lambs.

The percentage of respondents using each monitoring method as part of their nematode control program for (a) N. battus and (b) GIN control, and the total number of drenches typically administered to lambs per year (note, several respondents used multiple monitoring methods).

The monitoring method used to determine the timing of anthelmintic treatment was not found to be strongly associated with the number of drenches typically administered to lambs each year for *N. battus* or GIN control (Figure 5.7). FEC monitoring for *N. battus* and GIN was associated with frequent anthelmintic treatments and live weight, ill thrift monitoring and the use of online risk maps were more commonly used by respondents administering fewer drenches however, the differences were minimal.

5.4.7 Quarantine

The majority of respondents (77%) routinely quarantined animals being brought onto farm, 7% of respondents never quarantined new and returning stock and a further 16% occasionally did so. The use of quarantine was statistically significantly higher in southern England and Wales compared with northern England and Scotland (χ^2= 7.4, d.f. = 2, p= 0.03) however, the difference between individual regions was not statistically significant (χ^2= 21.6, d.f. = 14, p= 0.09) (Figure 5.8). Routine quarantine of new and returning stock was greatest in Wales, North Scotland and the southern regions of England. Quarantine usage was more varied in South Scotland and North England with a larger proportion of respondents reporting no quarantine practice or selected quarantine of replacement breeding stock only.

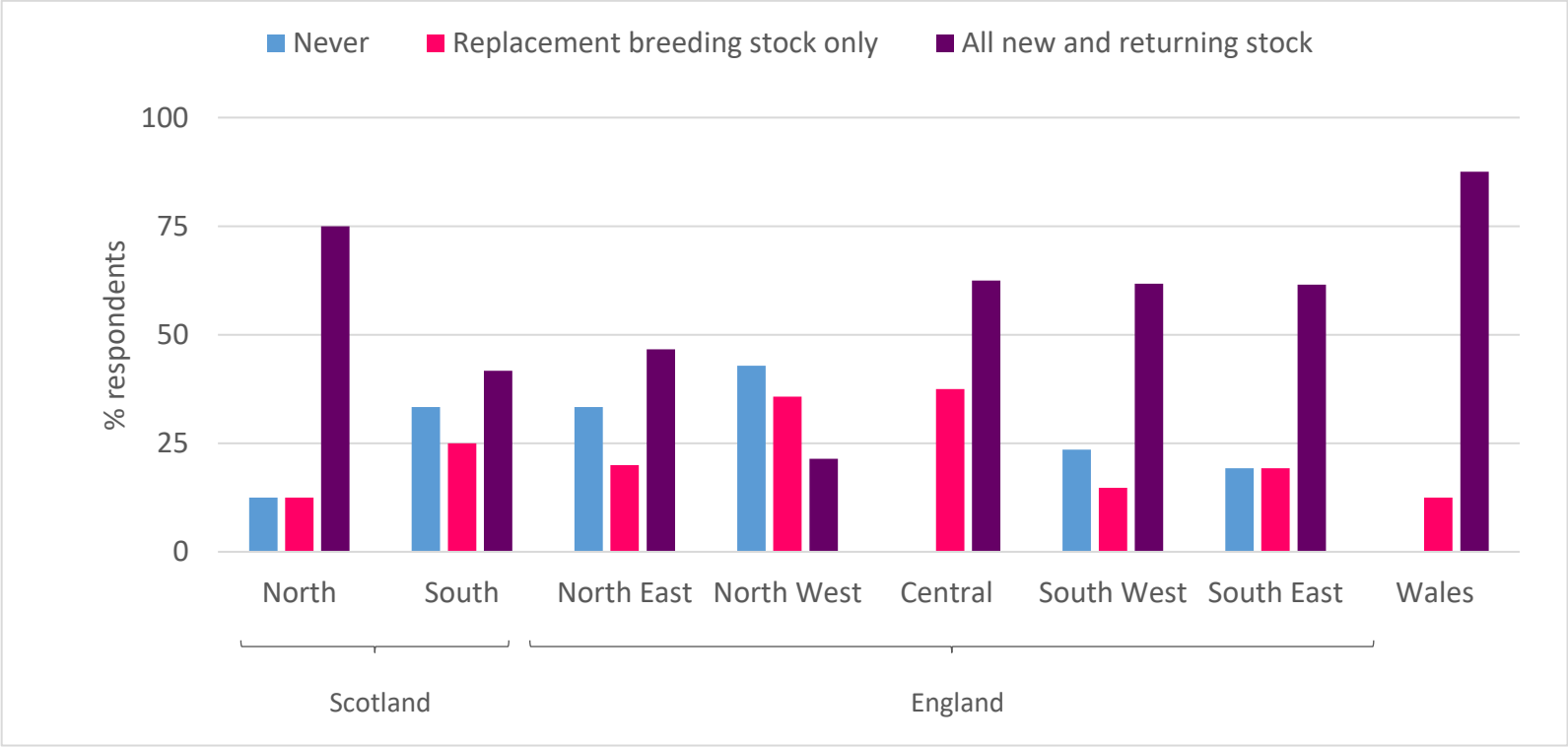


Figure 5.8. Quarantine practice employed by UK region.

Proportion of respondents employing some form of quarantine for all incoming stock (purple), replacement breeding stock only (pink) or not employing quarantine for any incoming stock (blue).

Quarantine practice varied greatly between respondents in both the anthelmintic treatment used and the length of time that incoming stock was isolated from the existing flock. A total of 23% of the respondents who quarantined animals on farm, followed SCOPS best practice guidelines. The guidelines recommend that all new and returning stock being brought onto farm should be yarded for 24 hrs, treated using at least one of the new anthelmintic compounds (4-AD or 5-SI) then kept separate from existing stock for a minimum of 21 days (Abbott et al., 2012). The use of SCOPS recommended quarantine practice was statistically significantly higher in the south of England than in the north of England and Scotland (χ^2= 5.2, d.f.= 1, p= 0.02); 21% of respondents in the South employed SCOPS recommended quarantine strategies compared to only 6% in the North. No statistically significant difference was observed between individual regions (χ^2= 8.4, d.f. = 7, p= 0.3).

The majority of respondents (74%) treated with a single anthelmintic class during quarantine; predominantly using ML (33%) or AD (37%). BZ was administered by 21% of respondents using a single quarantine treatment. LV and SI were not commonly used as single quarantine treatments (3% and 5% respectively).

Of the respondents who used multiple anthelmintic classes during quarantine, 32% included a novel agent (AD or SI). ML was the most common quarantine drench included in multiple class treatments (85%). Around half of respondents used LV and AD (50% and 41% respectively) and around ¼ included BZ (24%), few respondents used SI (15%).

The anthelmintic class used in quarantine treatment varied regionally. BZ-compounds were more commonly used in the north of England and Scotland than in southern regions (χ^2= 10.2, d.f. = 1, p= 0.001) and the opposite was found for AD use (χ^2= 8.9, d.f. = 1, p= 0.003).

The length of time new and returning stock were isolated from the main flock during quarantine varied considerably between farms from 0 to 84 days (mean±SEM; 25.6±3.8).

Animals were given a quarantine treatment then immediately introduced to the main flock by 9% of respondents. Duration of isolation was not found to vary geographically (χ^2= 6.6, d.f. = 7, p= 0.5) or be linked with the use of single or multiple anthelmintic classes during quarantine treatment (χ^2= 3.3, d.f.= 1, p= 0.07).

5.5 Discussion

The aim of the questionnaire study was to gather information on farmers' perceptions of *N. battus* disease and to investigate how control measures and general farm management practices vary throughout the UK. Stark differences were identified in the perceived disease severity, timing and sheep management North to South. Respondents from North England and Scotland observed changes in the severity of disease and overall higher prevalence of scouring and lamb losses attributed to *N. battus* infection in recent years. Subclinical disease and changes in the timing of *N. battus* were more commonly perceived in the south, this may be due to a combination of differences in environmental conditions, disease monitoring practices; greater uptake of faecal egg counting in the south or frequent reliance on yearly prophylactic treatment in the north.

Traditionally, *N. battus* was described as a spring disease of young lambs and this remains the most common time for infection in North England and Scotland. However, the timing of *N. battus* infection in southern regions was found to be more varied, with reports throughout the year, including a significant number of respondents observing disease in autumn and winter. Models of expected hatch date of *N. battus* eggs based on climatic conditions predicted earlier hatching in the South due to earlier spring temperature rises (Gethings et al., 2015; van Dijk et al., 2008). Lambing date was found to be consistent across regions, peaking in March to April. The more varied timing of *N. battus* predicted in the south

therefore suggests that a mismatch between egg hatching and grazing of susceptible lambs may be possible as previously suggested by van Dijk *et al.* (2008) and Gethings *et al.* (2015).

The severity of *N. battus* disease observed by respondents reflected the varied timing of infection. More severe signs were observed in Scotland and the North of England where spring infection was common and more subclinical infection was reported in the South. These findings agree with historic and current parasite surveillance data which show a trend towards higher incidence of *Nematodirosis* diagnoses and *N. battus* positive faecal samples submitted from Scotland, particularly in spring compared to other regions of the UK (van Dijk et al., 2008). Fewer diagnoses were recorded from the South West of England compared to the North and encompassed more autumn disease. The APHA data from recent years also highlighted an autumn peak in Welsh samples which was not reflected in the respondent's observations of disease in the current study. The lack of observation may be due to low level infection as APHA data was plotted at the observation level, i.e. all submissions which were positive for *N. battus*, no threshold of number of eggs per gram of faeces was used therefore points include both high and low egg count results. Surveillance data also relies on farmers submitting samples for diagnosis which will likely only occur in the case of anomalies.

Respondent's observations of disease severity suggest that the typical spring to spring transmission route remains the most dangerous for lambs and is arguably the most successful strategy for the parasite. The observed difference in timing of disease from North to South could be associated with climatic factors. It has been hypothesised that the varied timing of *N. battus* infection in the South could be explained by the pattern of spring temperatures; Scotland typically observe a slow increase in spring temperature, allowing eggs ample time to hatch whereas temperatures increase more quickly in the South, therefore a proportion of the eggs may not have time to hatch before temperatures rise through the upper threshold for hatching, eggs then subsequently hatch in autumn when the

temperature re-enters the hatching range (van Dijk et al., 2008; van Dijk and Morgan, 2008). Van Dijk *et al.* (2008) suggested that the spring transmission route may be less important in southern climates, increasing the likelihood of autumn transmission and the data collected here provides evidence to support this hypothesis.

Farm management practices varied North to South, particularly the grazing strategy of lambs and the method used to monitor the need for treatment, both of which could have a significant influence on *N. battus* epidemiology, altering the availability of suitable hosts and the timing and frequency of treatment. Grazing strategies involving the frequent movement of lambs, such as rotational systems, were found to be the most common overall, particularly in the South. Set stocking was generally favoured in the North. As *N. battus* was reported throughout the year in the South, the varied availability of hosts may interrupt transmission. Reduced disease severity in the South compared to Scotland (van Dijk et al., 2008) may result from restricted host availability or high UV levels in summer reducing larval survival time on pasture (van Dijk et al., 2009). Grazing strategy was also associated with the number of years respondents had been farming at their current address therefore, may be linked with likelihood to adopt novel practices, as suggested by a recent attitudinal survey (Jack et al., 2017).

Respondents also reported differences in the method of monitoring disease to determine optimal treatment timing with a greater reliance on faecal egg counting in the South. FEC is not recommended for monitoring *N. battus*, as severe symptoms can occur before eggs appear in faeces due to the intestinal damage caused by establishment of juvenile *N. battus* in the gut (Coop et al., 1973). Acute infection typically self-regulates with the expulsion of adult worms through a hypersensitivity reaction shortly after high *N. battus* egg counts (Mapes and Coop, 1972; Martin and Lee, 1976). Treatments based on egg count will therefore likely miss the optimal time, resulting in production losses from acute disease.

Uptake of FEC to inform the timing of anthelmintic treatment to control *N. battus* may have been driven by the reduction in acute *Nematodirosis* observed in the south. FEC may be useful in diagnosing low level infection in lambs later in the season, for example, where the spring egg hatch has been interrupted (van Dijk and Morgan, 2008). Higher reports of subclinical infection in the South than the North may also be due to FEC uptake, diagnosing low level infection which would otherwise be missed.

As shown by the APHA surveillance data (Figure 5.5), using FEC monitoring for *N. battus* would likely induce regional differences in treatment frequency and the timing of their application; triggering earlier spring treatment in the South and additional treatments in autumn. The timing and frequency of anthelmintic treatments have a significant impact on population dynamics and the selection pressure placed on the parasites. The maintenance of refugia is strongly influenced by the timing of treatment and pasture contamination and has been highlighted as a key factor in reducing the rate of AR development in other strongyle nematode species (van Wyk, 2001). However, the existence and influence of refugia in *N. battus* is difficult to quantify given the significant differences in life history traits between the species. It could be hypothesised that *N. battus* populations which are active throughout the year e.g. as observed in the South of England, may have a greater refugia compared with populations predominantly transmitted spring to spring. Only a proportion of the varied hatching population would be active at any one time therefore each anthelmintic treatment exerts selection pressure on only a proportion of the population. Similarly, the timing of treatment has a significant influence on the level and selection of pasture contamination laid down for the following year. The impact of autumn treatment may not be as significant as the altered timing of spring treatments given that anthelmintic control of strongyle nematodes is common at this time and BZ compounds are less commonly used later in the season. Over half of respondents reported using BZ compounds to control strongyle nematodes however, largely in combination with other anthelmintic

classes. Additional treatments in autumn using anthelmintic classes other than BZ could reduce the rate of development of BZ-resistance in those populations by clearing individuals carrying the resistant genotype.

In the current study, FEC monitoring was more commonly used on farms which typically administered 5-6 drenches per year to lambs than those which administer 1-2 drenches per year. This appears counter-intuitive however, information on parasite contamination level or the thresholds used for treatment based on FEC was not collected. The association between FEC monitoring in lambs and increased treatment frequency may be skewed by individuals administering treatments following the observation of few eggs. Faecal egg count monitoring throughout the year to control all nematode species has been shown to create a potential cost/benefit of over £5500 on a large farm (~1900 ewes). However, training in the appropriate use of FEC and setting thresholds for treatment is essential to maximise the benefit of this evidence-based approach and reduce selection pressure on the parasite population. Regular FEC monitoring would also likely highlight anthelmintic failure more quickly than other monitoring methods. A study of the production cost of un-detected anthelmintic resistance identified a 10.4% reduction in carcass value over a season with lambs finishing on average 17 days later resulting in financial losses which out-weighed the cost of routine efficacy testing (Miller et al., 2012).

The second most commonly reported monitoring method was the observation of ill thrift in lambs which, again may miss the optimal timepoint for treatment as production losses typically occur prior to observable ill thrift in animals. Despite effective treatment upon the observation of disease symptoms and sufficient feeding etc. for the remainder of the grazing season, the check in growth from *N. battus* infection in lambs has been shown to be non-recoverable (ROWLANDS and Probert, 1972), resulting in lower liveweight and therefore decreased profits from a commercial farming viewpoint. Respondents relying on ill thrift to

determine treatment timings also administered the second highest number of treatments per year. Highlighting the increased anthelmintic usage and production losses associated with ill thrift monitoring (Kenyon et al., 2013) to the farming community may reduce dependence on this method and increase the uptake of evidence-based approaches.

Prophylactic treatments, administered at the same time each year, were employed by around 17% of respondents, predominantly in North England and Scotland, perhaps due to variation in farming systems throughout the country and the different management requirements and observation opportunities afforded by each. Historically, *N. battus* control was based around yearly blanket treatments in spring however, the changing epidemiology of this species and the influence of climate change has resulted in variation in the timing of egg hatching. Gethings *et al*. (2015) predicted the first availability of *N. battus* larvae in different years by modelling temperature and the impact of chilling on egg hatching (Figure 5.9) and found that the predicted egg hatch varied significantly between years. The predicted variability therefore suggests that treatments administered based on calendar dates would be unlikely to be effective. However, the predicted hatch date of eggs in Scotland was found to be more stable in the face of climate change compared to the South of England therefore perhaps explaining the continued favour of prophylactic treatment in the North.

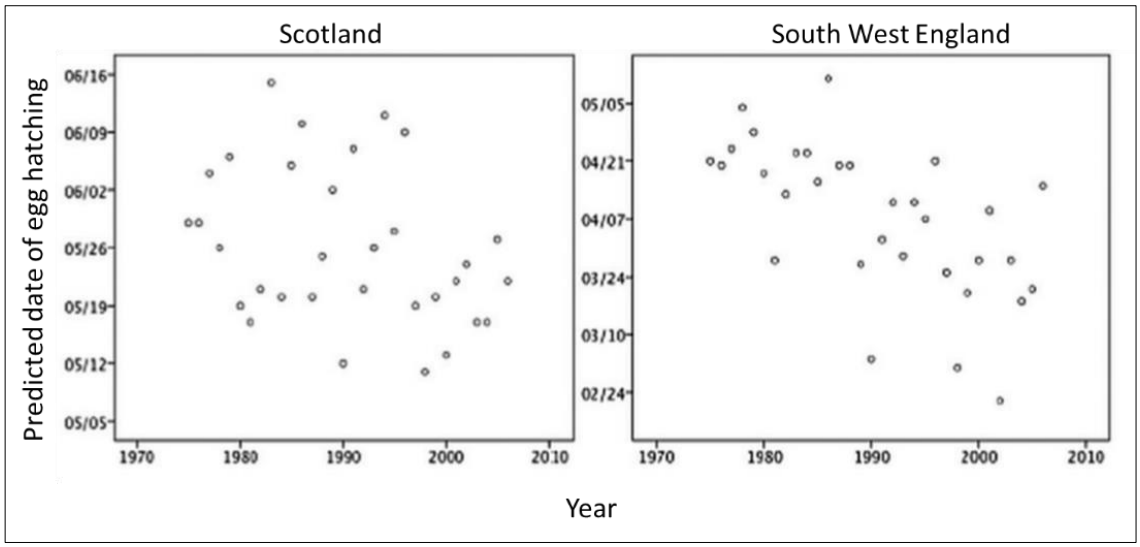


Figure 5.9. Predicted date of first N. battus larvae available on pasture each year.

Regional predicted egg hatching dates based on a mechanistic model of temperature and the impact of winter chilling on N. battus from 1970 to 2010. Figure adapted from Gethings et al. 2015 with the permission of Cambridge university press.

Online risk maps predict the timing of *N. battus* eggs hatching in spring based on local meteorological data (Stubbings, 2018) therefore allowing users to target anthelmintic treatment or altered grazing strategies to high risk timepoints. Around a third of respondents reported using online risk maps to determine the optimal treatment timing. The forecast utilises weather station data in a model which predicts when environmental conditions will be optimal for *N. battus* eggs to hatch on pasture based on previous research, adapting to annual climatic variation. The use of online tools is promising, indicating that a significant proportion of respondents have engaged with best practice advice. Regional variation in uptake identified could be used to target knowledge exchange or training initiatives to encourage the use of this technology throughout the country.

The anthelmintic class of choice for the control of *N. battus* has been the benzimidazoles (BZ) since they were first licenced for use in livestock in the 1960's (Brown et al., 1961) and the

results of the current questionnaire show that these compounds remain the most common. Despite increasing reports of autumn *Nematodirosis*, acute spring infection in young lambs continues to provide the most significant disease threat and, given blanket BZ treatments administered to lambs at this time, it represents the greatest selection pressure for AR in *N. battus*. Treatments administered throughout the grazing season to control *N. battus* in the south will exert a selection pressure on only a small proportion of the population therefore suggesting that BZ-resistance risk may be higher in populations which rely on spring to spring transmission, as are common in the North. Interruption of spring-to-spring transmission through alternate grazing strategies was shown to reduce *N. battus* contamination on pasture (Black, 1959; Boag and Thomas, 1975) and has been advocated for many years. However, repeated grazing of fields by 1-3 month old lambs each spring was employed by almost all respondents in the current study, with few having re-seeded this land within the previous five years to reduce pasture contamination. Increasing the uptake of avoidance grazing strategies could provide a significant benefit, particularly in Scotland and North England where spring-to-spring transmission is common and responsible for severe disease outbreaks. The long-term influence of such strategies is unclear though as interruption of transmission in spring may put a selective pressure on the parasite to hatch at different times of year although the disease threat from autumn hatched larvae would be expected to be lower than that posed by high spring challenge.

The use of other drug classes for the control of *N. battus* appears to have increased within recent years. The use of combinations of anthelmintics which included BZ were common, likely in response to the lengthening seasonality of the species and subsequent co-infection with other strongyle nematode species. Additionally, public reports of the emergence of resistance in this species and high BZ-resistance in other nematode species (Thomas et al., 2015) may have influenced anthelmintic choices or veterinary advice. The use of BZ-compounds early in the season is still advocated (Abbott et al., 2012) to prevent additional

selection pressure for other GIN species which may be present in small numbers at that time, protecting the other anthelmintic classes which will be required throughout summer and autumn. The two new anthelmintic compounds AD and SI were not commonly used for *N. battus* control, this was as expected given the general high efficacy of the older anthelmintic classes against *N. battus* and the high cost of the new compounds by comparison.

More than ¾ of respondents in the current study reported implementing a quarantine strategy for new and returning stock however, the treatments administered and the length of isolation varied considerably. SCOPS guidelines advise that all incoming animals should be treated with at least one of the novel anthelmintics (AD or SI) and isolated for at least 21 days before being introduced to the existing flock which was implemented by only 20% of those quarantining animals (Abbott et al., 2012). The majority of respondents treated with a single active during quarantine. More than 1/5th of respondents in the current study used a single benzimidazole treatment during quarantine which, given current estimations of resistance on UK sheep farms (Thomas et al., 2015), would be unlikely to be effective. Isolation time varied greatly between farms from immediate introduction to separation for a full grazing season. A study of English farmers by the Department for environment, food and rural affairs (DEFRA) in 2014 revealed that 52% of farmers who did not isolate incoming stock during quarantine believed that it was not required and a further 19% did not believe that it would make a difference (Department for Environment, 2014). The DEFRA study included farmers from all agricultural industries and was not broken down by sector so cannot be directly compared with the sheep farming results discussed in this chapter however, it does highlight the need for future initiatives to illustrate the benefit of effective quarantine.

5.6 Conclusions

The findings of the current study suggest that the *N. battus* population dynamics are likely to differ significantly between populations from North to South, possibly due to the impact of climatic variation and differential management practices on parasite epidemiology. Respondents in Scotland and North England reported infection primarily during spring and early summer suggesting a typical spring-to-spring transmission pattern, resulting in acute disease in young lambs. Respondents from the South reported infection throughout the year with significant peaks in autumn which may indicate a more varied hatching pattern. The management and control information discussed in this chapter provides a basis from which to explore the relative impact of different husbandry practices on the recent changes in *N. battus* to inform the design of future sustainable control measures. The current study also highlighted a number of knowledge exchange opportunities. Simple control measures such as avoiding grazing lambs on the same pasture each spring would be easy to implement on some farms and could significantly reduce *Nematodirosis* in heavily infected regions. Avoidance grazing has been promoted for many years with the benefit of these strategies being demonstrated as far back as 1959 (Black, 1959). The low uptake of simple practices is likely due to ineffective dissemination of information and demonstration of the potential benefits. Some recommendations have been widely adopted, such as online risk maps which is promising however, the incorrect use of practices such as FEC and live weight-gain for monitoring *N. battus* infection in lambs indicate that some of the messages may be confused. The findings of this questionnaire study could be used to identify knowledge gaps and target training events to the regions where uptake is low.

6 Drivers and barriers for the selection of benzimidazole resistance in *Nematodirus battus*

6.1 Abstract

Anthelmintic resistance is widespread in many livestock nematode species globally. Sustainable parasite control therefore relies upon understanding the mechanisms and drivers of the development and dissemination of resistance. Despite the repeated use of whole-flock benzimidazole treatments to control *Nematodirus battus* in young lambs each spring, resistance has only recently emerged in this species. The prevalence of resistance mutations (primarily F200Y, β-tubulin isotype 1 gene) remains low throughout UK sheep flocks with apparent focal regions of high resistant allele frequency. General linear mixed model (GLMM) analysis was used to explore potential drivers associated with F200Y allele frequency in *N. battus* populations using farm management data collected by questionnaire in addition to environmental factors including temperature, precipitation and relative humidity extracted from Met Office record data. The results of the best fitted model indicated that quarantine was the main protective factor and that set stocked grazing and reseeding of heavily contaminated pastures were the main risk factors associated with higher prevalence of the F200Y mutation for BZ resistance on farm. Not following SCOPS recommendations on quarantine practice increased the odds of finding F200Y resistant alleles within the *N. battus* population on farm by 33 times (OR 0.03, 95% CI 0.003, 0.17, $p<0.001$) and suggested that the maintenance of refugia and reinfection of 'clean' pasture may be central to the development or lack of development of BZ-resistance in *N. battus* (reseeding of contaminated pasture; OR 6634, 95% CI 39.25, $2.0x10^6$, $p<0.001$).

6.2 Introduction

Nematodirus battus had up to recently been believed to be refractory to benzimidazole resistance. The initial case of BZ-resistance in *N. battus* was identified in 2010 (Mitchell et al., 2011) and the single nucleotide polymorphism (SNP) F200Y in the β-tubulin isotype 1 gene, previously associated with BZ-resistance in other strongyle species, was found to confer resistance in this species also. The genotyping survey of 265 UK *N. battus* populations (chapter 2) identified F200Y in *N. battus* populations throughout the UK, albeit at a low allele frequency overall (~2%). The resistant allele was identified in around one in four of the populations examined with one confirmed and additional suspected focal regions containing several farms with high resistant allele frequency.

In several other economically important strongyle nematode species, such as *Teladorsagia circumcincta* and *Haemonchus contortus*, AR is widespread, with multiple resistance to the three traditional broad-spectrum anthelmintic classes repeatedly identified (Keane et al., 2014; Ramunke et al., 2016; Thomas et al., 2015). Limited reports of resistance against the newer classes; 4-AD and 5-SI (Mederos et al., 2014; Scott et al., 2013; van den Brom et al., 2015) have also started to emerge. Many studies have investigated management strategies as potential risk factors for the development of anthelmintic resistance in strongyle nematode species. The frequency and timing of anthelmintic treatments has been highlighted as being significantly associated with the presence of anthelmintic resistance in the study population in multiple studies (Falzon et al., 2014; Suarez and Cristel, 2014; Suter et al., 2004; Vadlejch et al., 2014). The frequency of treatment relates directly to the level of selection pressure placed on the parasite population. However, the timing of treatment influences the availability of parasites to re-infect animals post-treatment i.e. the maintenance of refugia, recognised as one of the key determinants of the rate of AR development in trichostrongylid species. Refugia refers to the parasite populations which are

not exposed to anthelmintic pressure and which are available to contribute to the gene pool of parasites re-infecting animals following treatment. The importance of refugia and methods for maximising the susceptible population have been widely reported for strongyle species (Kenyon et al., 2009; Leathwick et al., 2009; Leathwick et al., 2008; van Wyk, 2001). However, due to the significant differences in epidemiology and life history traits between *N. battus* and other GIN species, the mechanisms, importance or existence of refugia in this species remains unclear. The key factor in refugia is maintenance of a sizable susceptible population to dilute any resistant individuals which survive anthelmintic treatment. Several factors have been shown to reduce refugia of strongyle species, including frequent anthelmintic treatments, treatments administered at time points of low parasite abundance (Calvete et al., 2012), moving animals to low contamination pasture immediately after treatment and climatic conditions which are not optimal for larval survival on pasture (Cawthorne and Whitehead, 1983; Jackson and Coop, 2000; Taylor and Hunt, 1988). Strategies have been devised to maximise refugia during the summer grazing season, largely based around leaving a proportion of animals untreated to increase the dilution of any resistant parasites surviving treatment (Besier, 2008; Kenyon et al., 2009). However, as a high proportion of animals may rapidly become severely ill after mass hatches of infective larvae, these practices are not suitable for *N. battus* control. During mass infection in spring, the majority of the *N. battus* population is active at once however, larvae not ingested quickly die on pasture (Thomas and Stevens, 1956; van Dijk et al., 2009; van Dijk and Morgan, 2008) and those established are often rapidly eliminated due to hypersensitivity reactions triggered by the extremely high challenge afforded in spring following mass hatch events. The interaction, if any, between spring infections and those sub-populations which hatch later in the season is unknown therefore the size and dynamics of “refugia” in *N. battus* cannot be determined at present.

The aim of the current study was to investigate potential drivers of the development and dissemination of BZ-resistance in *N. battus* by testing for associations between resistant allele frequency, farm management practices and environmental factors. BZ-resistance is currently at an early stage in *N. battus*, understanding the drivers associated with the emergence and spread of anthelmintic resistance in this species may provide an opportunity to slow the development of resistance in this species. Protecting BZ compounds for use in the future will allow for continued safe treatment of young stock whilst preventing further selection pressure of the other anthelmintic classes.

In line with other strongyle species, we hypothesise that the development of BZ-resistance in *N. battus* will be influenced by the frequency of anthelmintic treatment and the implementation of effective quarantine to prevent the introduction of anthelmintic resistant nematodes onto the farm.

6.3 Methods

6.3.1 Data Sources

The F200Y allele frequency of 282 field populations of *N. battus* from throughout the UK were analysed using a pyrosequencing assay. See chapter 2 for details of sample collection, preparation, DNA extraction and pyrosequencing methodology. Farm management data were collected by online questionnaire, completed by 87 of the farmers who provided samples for the genotyping survey. Details of questionnaire development and dissemination are explained in chapter 5 and raw questionnaire data is detailed in appendix 6.

Climatic data for risk factor analysis was obtained from the Met office DataPoint (Met Office, 2018). The data was collected from a large number of weather stations and sensors across the UK and the information was downloaded as an interpolated data layer. Data layers were

downloaded for temperature (minimum and maximum for each month of the year and mean annual values), precipitation (mm, annual average), ground frost (average annual days), sun hours (average number of hours of sunshine) and relative humidity (%). Evapotranspiration (mm, annual mean) data was sourced from the 'Atlas of the biosphere' (Centre for Sustainability and the Global Environment, 2018) and elevation (meters above sea level) at the point of the farmhouse was also included as a rough approximation of elevation of the farm in general, data obtained from DIVA-GIS (Annon., 2018). All environmental data was mapped using open source qGIS software (Las Palmas version 2.18), the farm positions were then mapped on top of the data and values from each data layer were extracted at the farm locations. Table 6.1 contains a summary of the covariates included in model development and detail of how each factor was coded.

6.3.2 Generalised linear mixed model screening analysis and model fitting

A generalised linear mixed model (GLMM) with binomial outcome; the number of resistant alleles out of the total number of alleles identified on each farm, was fitted with a logistic link. In order to account for the expected clustering in the data, farm was fitted as a random effect.

Univariate analysis was conducted to identify potential risk factors for inclusion in the fitted models; individual GLMMs were constructed as described above, including each environmental, management and demographic factor (listed in Table 6.1) as fixed effects. Fixed effect factors with a p-value less than 0.2 (Table 6.3) were used to build the fitted risk factor model by a forward inclusion method. Coefficients were added to the model individually and retained if the factor was found to have a statistically significant impact at the 5% level. Multiple models were developed during the analysis based on the order in which coefficients were added. The GLMM method used incorporates factors in the order in which they were presented and does not retain data points with missing values. The data set

for each model was therefore determined by the factors included i.e. any farms/populations with a missing data point for any one of the coefficients incorporated in the model would not be included in the analysis. Once the model was fitted, each coefficient rejected during screening analysis was added to the model individually to test for significance and inclusion in the final model. A further model was constructed using a mixed approach to explore the impact of removing non-significant factors retained within the fitted models when the forward inclusion method was used. Working backwards from the fitted forward inclusion models, factors which had a p-value greater than 0.05 were removed one at a time until all remaining model covariates were statistically significant. Again, once the model was fitted, each coefficient rejected during screening analysis was added to the model individually to test for significance and inclusion in the final model.

Fitted models were compared using a chi-square test where possible by fitting all models to the sub-set of data used by the model requiring the least observations. Where models would not converge given the restricted data set, an F-test was performed to compare the extra-binomial variance assigned to the random effect in each fitted model.

A Kruskal Wallis test was also used to compare quarantine usage between farms with 0, <5% and >5% F200Y resistant allele frequency. All statistical analysis and model building were performed using R version 3.2.5.

Table 6.1. A list of the covariates included in GLMM analysis including the units of each factor and the coding method used (categorical, continuous or binary).

Factor	Coding
Farm demographics	
Longitude/latitude	Continuous
Farm elevation (m above sea level)	Continuous
Farm type (lowland/upland/hill)	Categorical
Predominant sheep breed	Categorical
Farming enterprise (breeding/ fattening or finishing/ both)	Categorical
Age of lambs sampled (week of the year)	Continuous
Peak lambing date (week of the year)	Continuous
Timing of sample collection (week of the year)	Continuous
Farmer perception of N. battus infection on farm: symptom severity (scouring and lamb losses/ scouring only/ subclinical/ not known to be present)	Categorical
Timing of *N. battus* symptoms (spring/ summer/ autumn/ winter)	Categorical
N. battus symptoms observed in autumn and winter	Binary (yes=1)
N. battus symptoms observed in spring only	Binary (yes=1)
Environmental factors	
Spring (March - May) minimum temperature (°C)	Continuous
Spring maximum temperature (°C)	Continuous
Autumn (September – November) minimum temperature (°C)	Continuous
Autumn maximum temperature (°C)	Continuous
Mean annual temperature (°C)	Continuous
Mean annual minimum temperature (°C)	Continuous
Mean annual maximum temperature (°C)	Continuous
Mean annual precipitation (mm)	Continuous
Mean annual days of ground frost (days)	Continuous
Mean hours of sunshine per month (hours)	Continuous
Mean evapotranspiration per month (mm)	Continuous
Farm management	
Primary grazing strategy employed for lambs (set stocking/ rotational grazing/ cellular grazing/ leader follower system)	Categorical
Co-grazing of lambs with other stock	Binary (yes=1)
Grazing land separate from the main farm (away grazing)	Binary (yes=1)
Supplementary feed provided for lambs	Binary (yes=1)
Are fields routinely grazed by 1-3 month old lambs each spring (i.e. development of 'high-risk' fields for *N. battus* infection)	Binary (yes=1)

Factor	Coding
Have 'high risk' fields been reseeded in the 5 years prior to the survey	Binary (yes=1)
Have 'high risk' fields been rested in the 5 years prior to the survey	Binary (yes=1)
Stocking density of lambs pre- and post- weaning (heads per acre)	Continuous
Are new or returning stock quarantined (no/yes/sometimes)	Categorical
Length of time stock are isolated for during quarantine (days)	Continuous
Anthelmintic class used as quarantine treatment	Categorical
Does typical quarantine practice align with SCOPS recommended practice	Binary (yes=1)
Anthelmintic treatments	
Mean annual number of anthelmintic treatments administered to lambs/ewes/rams (number of treatments)	Continuous
Anthelmintic class(s) used to control *N. battus* within the previous 12 months/5years	Categorical
Predominant anthelmintic class used to control strongyle species in the past 5 years	Categorical
Use of Ill thrift to determine timing of anthelmintic treatment in lambs/ewes/rams for the control of *N. battus*/GIN	Binary (yes=1)
Use of faecal egg counting to determine timing of anthelmintic treatment in lambs/ewes/rams for the control of *N. battus*/GIN	Binary (yes=1)
Use of veterinary plans to determine timing of anthelmintic treatment in lambs/ewes/rams for the control of *N. battus*/GIN	Binary (yes=1)
Use of online risk maps to determine timing of anthelmintic treatment in lambs/ewes/rams for the control of *N. battus*/GIN	Binary (yes=1)
Administer anthelmintic treatments to lambs/ewes/rams prophylactically (i.e. at the same time each year) for the control of *N. battus*/GIN	Binary (yes=1)
Administer anthelmintic treatments to ewes/rams at mating for the control of GIN	Binary (yes=1)
Administer anthelmintic treatments to ewes/rams at lambing for the control of GIN	Binary (yes=1)
Samples	
Time in faeces: between collection and egg extraction (days)	Continuous
Egg development time: between extraction and experimental set up (days)	Continuous
Total storage time (days in faeces + days of egg development) (days)	Continuous
N. battus/trichostrongylid eggs per gram in original sample (eggs per gram faeces)	Continuous

6.4 Results

Chapter 2 investigated the prevalence of SNPs associated with BZ-resistance in *N. battus*. F200Y was the most common SNP, identified in around one in four of the 284 UK populations tested (65 populations positive for F200Y), despite the high prevalence, resistant allele frequency was low overall; ~2.1 ±0.6% (mean ±SEM), range 0 to 93%. The mixed models were fed using data collected from both the genotyping study (chapter 2) and farm management data (chapter 5). The farm management questionnaire was completed by 87 study farms (with genotyping data), of those 37 populations both contained F200Y resistant alleles and had management data available; 25 populations with 1-5% F200Y and 12 populations with >5% resistant alleles; 44 populations had management data collected and no resistant alleles identified. Table 6.2 summarises the farm demographics of populations with F200Y allele frequencies of 0%, <5% and >5%.

Table 6.2. Summary of the farm demographics of farms included in the genotyping study.

Farm demographics data from those farms with both questionnaire data and genotyping results, divided by F200Y resistant allele frequency. The table details the percentage of respondents who gave each response and the mean ±standard error for the number of livestock kept.

Questionnaire question	**Response**	**F200Y resistant allele frequency**		
		0	<5%	>5%
Livestock kept (mean ±SEM)	Ewes	504 ±74	680 ±231	215 ±48
	Rams	19 ±5	16 ±4	7 ±1
	Lambs	688 ±108	983 ±328	329 ±81
	Cattle	113 ±33	116 ±37	78 ±48
Type of farm	Lowland	67	61	55
	Upland	21	30	27
	Hill	12	9	18
Farming enterprise	Commercial	67	83	64
	Pedigree	16	9	9
	both	16	9	27
Time farming at current address	0-5 years	12	17	9
	5-20 years	30	48	36
	20+ years	58	35	55
Sheep enterprise	Breeder	24	22	18
	Finisher (buying lambs in the summer for fattening)	12	4	0
	Both	63	74	82

6.4.1 Screening analysis

A total of 66 fixed effects were individually analysed by GLMM (12 environmental factors and 54 farm demographic and management variables) (Table 6.1). A total of nine fixed effects were found to be significantly associated with resistant allele frequency to the 5% level, a further eight were significant to the 10% level and an additional 11 to the 20% significance level (Table 6.3).

Table 6.3. Results of univariate screening GLMM analysis.

Results of univariable GLMM (fitted with a logit link) analyses with binomial outcome of the number of F200Y resistant alleles identified out of the total number of alleles identified on each farm. Results are presented as odds ratios, 95% confidence intervals and p-values (Wald tests). Factors included were found to be significantly associated with F200Y allele frequency at the 5% level (p<0.05), 10% level (p<0.1) and at the 20% level (p<0.2) which were used to build the risk factor models.

Factor	Odds ratio	95% CI		p-value
		lower	upper	
N. battus symptoms more severe over the previous 5 years	420.31	21.09	53530.13	0.0003
N. battus symptoms observed in spring only	7.46	1.63	39.25	0.0098
Lambs predominantly grazed on set stocked fields	3.73	1.08	13.57	0.034
Albex usage for the control of *N. battus*	1.61	1.07	2.45	0.0177
Peak lambing week	0.85	0.71	1.00	0.0472
3-ML compounds used for quarantine treatment	0.56	0.32	0.95	0.0306
Average number of anthelmintic treatments administered to ewes per annum	0.51	0.25	0.93	0.0339
Quarantine strategy following SCOPS recommendations	0.11	0.02	0.60	0.0142
Ill thrift monitoring to determine the timing of anthelmintic treatment to control GIN in ewes	0.09	0.01	0.74	0.0332
Number of rams kept	0.97	0.93	1.00	0.0888
Stocking density of lambs post weaning	0.87	0.74	1.01	0.0746

Stocking density of ewes and lambs pre-weaning	0.87	0.73	1.00	0.0764
5-SI compound used for quarantine treatment	0.59	0.26	1.01	0.0835
Sporadic co-grazing of lambs with adult beef cattle	0.48	0.21	1.00	0.0538
FEC monitoring to determine the timing of anthelmintic treatment for the control of GIN in lambs	0.26	0.06	1.09	0.0623
Reseeding of high risk pasture*	0.18	0.05	0.67	0.0677
N. battus symptoms typically observed in autumn/winter	0.09	0.01	0.95	0.053
Co-grazing of lambs with young beef cattle post-weaning	7.57	0.47	127.36	0.135
N. battus anthelmintic treatment failure in previous 5 years	2.42	0.53	10.25	0.1241
Autumn grazing of high risk pasture by lambs*	1.47	0.87	2.53	0.14
Typical length of isolation during quarantine	0.96	0.91	1.01	0.123
Supplementary feeding of lambs	0.73	0.43	1.17	0.197
Ill thrift monitoring to determine the timing of anthelmintic treatment to control GIN in lambs	0.33	0.07	1.42	0.137
Anthelmintic treatment of ewes at mating to control GIN	0.31	0.06	1.61	0.157
Resting of high risk fields*	0.29	0.06	1.36	0.105
N. battus symptoms observed in ewe-lambs	0.27	0.03	1.69	0.1838
Typical number of lambs per year	0.02	1.9×10^{-5}	2.67	0.14
Number of breeding ewes kept	0.01	2.5×10^{-7}	2.51	0.183

*High risk pasture defined as land routinely grazed by lambs aged less than three months old each spring.

Screening analysis highlighted increased perceived severity of disease symptoms in the five years prior to the survey as being statistically significantly associated with F200Y allele frequency (OR 420.31, 95% CI 21.09, 53530.13). The wide confidence intervals suggest that this correlation was based on few farm populations.

The timing of observed *N. battus* symptoms on farm was found to be significantly associated with F200Y frequency (Figure 6.1). Resistant alleles were positively associated with the observation of symptoms exclusively in spring (OR 7.46, 95% CI 1.63, 39.25) and negatively linked with the reports of autumn and winter symptoms (OR 0.09, 95% CI 0.01, 0.95).

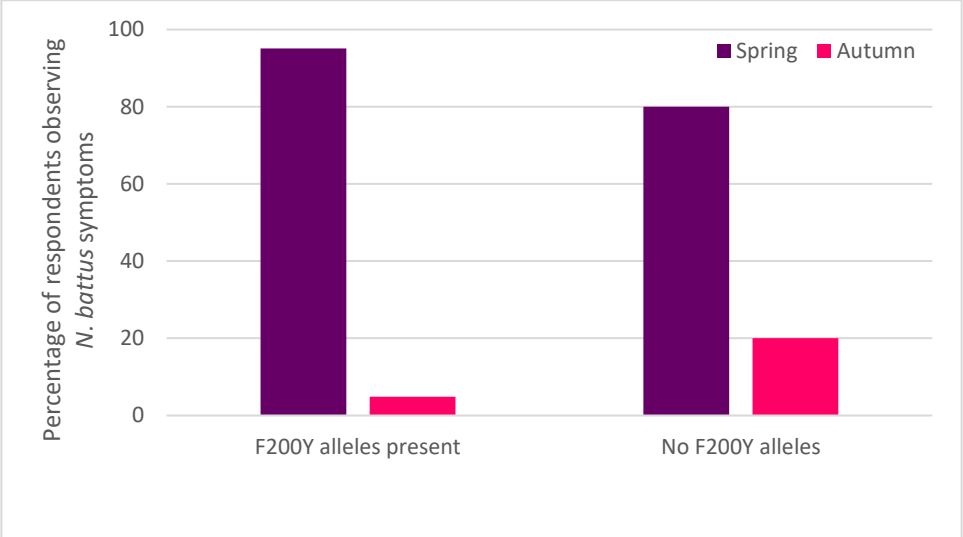


Figure 6.1. Observation of N. battus symptoms on farms with and without F200Y resistant alleles present. Percentage of respondents observing N. battus symptoms exclusively in spring compared with those observing autumn symptoms of disease, divided by the presence of F200Y resistant alleles.

Several factors relating to quarantine practice were retained during screening analysis. Figure 6.2 illustrates the proportion of respondents who routinely administer a quarantine treatment to new and returning stock being brought onto farm. Approximately half of respondents routinely quarantined all incoming stock. The implementation of some form of quarantine was did not vary significantly with resistant allele frequency (X^2=1.95, d.f. = 2, p= 0.38).

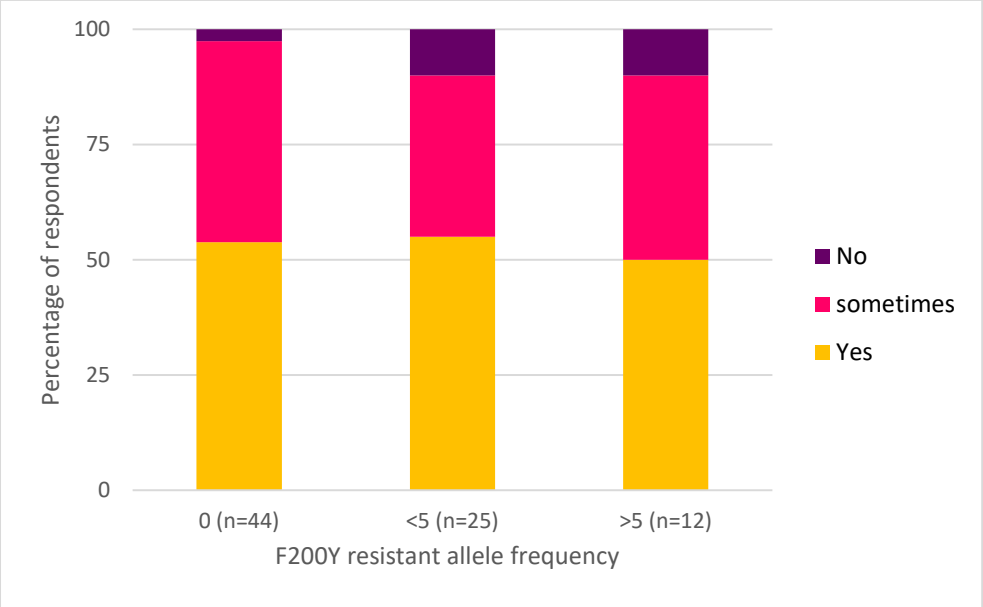


Figure 6.2. Quarantine practice on farms with varying F200Y resistant allele frequency.

The level of quarantine is represented by bar colour; routine quarantine treatment administered of all new and returning stock (yellow), quarantine practices used sometimes/ a proportion of incoming stock quarantined treated (pink) and never quarantine treat stock (purple). (n= number of respondents).

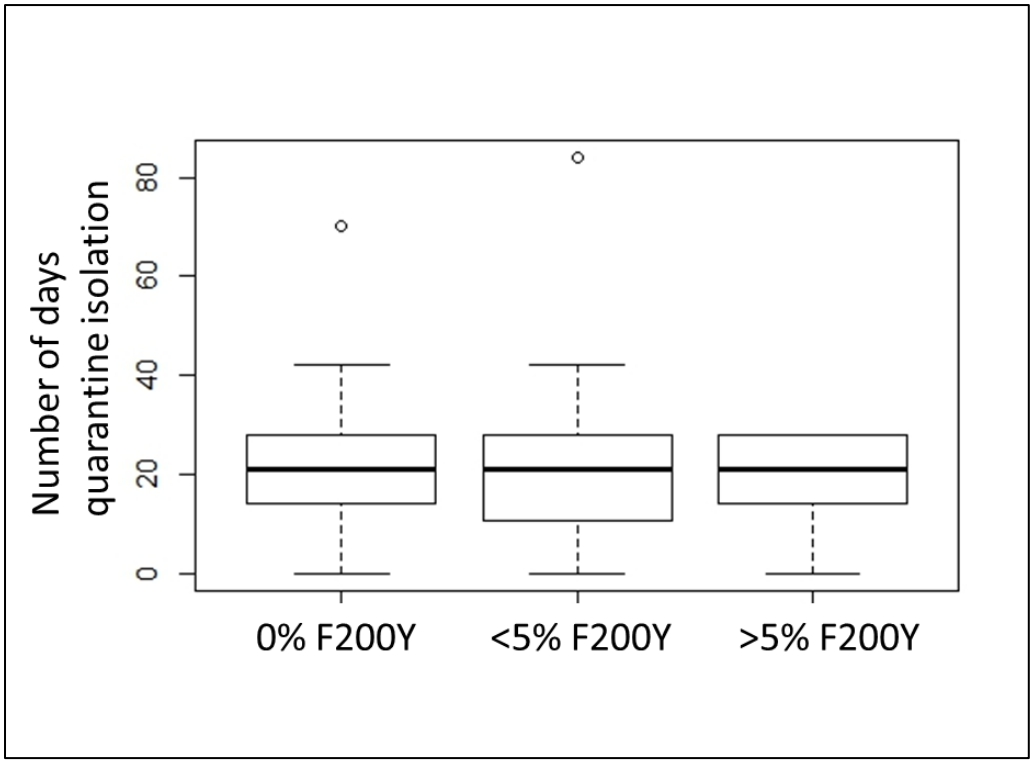


Figure 6.3. Quarantine isolation time on farms with varying F200Y resistant allele frequency.

Box and whiskers plot of number of days new and returning stock are isolated from the existing flock during quarantine on farms with N. battus *populations containing 0% (n=44), <5% (n=25) and >5% (n=12) F200Y resistant allele frequency.*

Additional questions regarding the quarantine practices used by respondents highlighted the variability in practices used throughout the UK, 20% of respondents employed quarantine practices which followed SCOPS recommendations. GLMM screening analysis found that following the SCOPS guidelines on quarantine was significantly associated with reduced BZ-resistant alleles identified on farm (OR 0.11, 95% CI 0.02, 0.60). The length of time new and returning animals were kept isolated from the main flock varied significantly between respondents from immediate introduction to the main flock up to 6 months separate however, this variation appeared to be independent of F200Y allele frequency (Figure 6.3). The length of separation was retained during screening analysis but was not found to be

statistically significantly associated with resistant allele frequency at the 5% level by GLMM analysis (OR 0.96, 95% CI 0.91, 1.01).

Figure 6.4 details the anthelmintic classes used in quarantine treatments by respondents with varying F200Y resistant allele frequencies on farm. No statistically significant association was identified between F200Y resistant allele frequency and the use of BZ compounds during quarantine however, screening analysis did retain the use of macrocyclic lactones (3-ML) which was less common with increasing F200Y allele frequency (OR 0.56, 95% CI 0.32, 0.95).

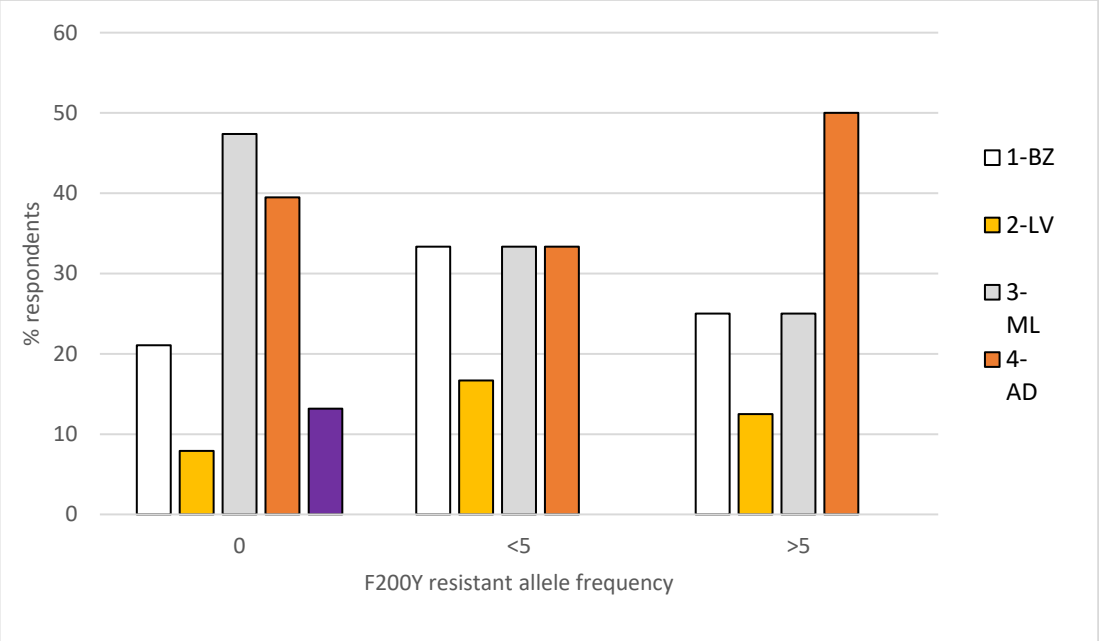


Figure 6.4. Anthelmintic class used for quarantine treatment on farms with varying F200Y resistant allele frequency.

The percentage of respondents from farms with 0% (n=44), <5% (n=25) and >5% (n=12) F200Y resistant allele frequency N. battus populations using each anthelmintic class as quarantine treatments.

Anthelmintic control measures including anthelmintic class of choice over the previous 12 months or 5 years did not appear to vary significantly with F200Y allele frequency. The number of treatments typically administered to lambs each year and the monitoring methods used for determining the timing of treatment were not found to be statistically

significant during screening analysis however, the number of treatments administered to ewes and the use of ill thrift monitoring in adult sheep were highlighted as being potentially important (OR 0.51, 95% CI 0.25, 0.93 and OR 0.09, 95% CI 0.01, 0.74 respectively).

Grazing and grassland management was also highlighted by initial GLMM screening; set stocked grazing was associated with increased F200Y resistant allele frequency compared to farms employing rotational, cellular or leader/follower systems (OR 3.73, 95% CI 1.08, 13.57). No statistically significant association was identified between resistant allele frequencies and co-grazing or reseeding of 'high risk' fields (i.e. fields routinely grazed by 1-3 month old lambs each spring) at the 5% level; however, these were retained during screening analysis for model building at the 20% and 10% significance level respectively. No respondents with >5% resistant alleles had reseeded 'high risk' fields in the five years prior to the survey compared with 15% of farms with <5% F200Y allele frequency and 29% of farms with no resistant alleles identified.

6.4.2 Fitted multivariable models

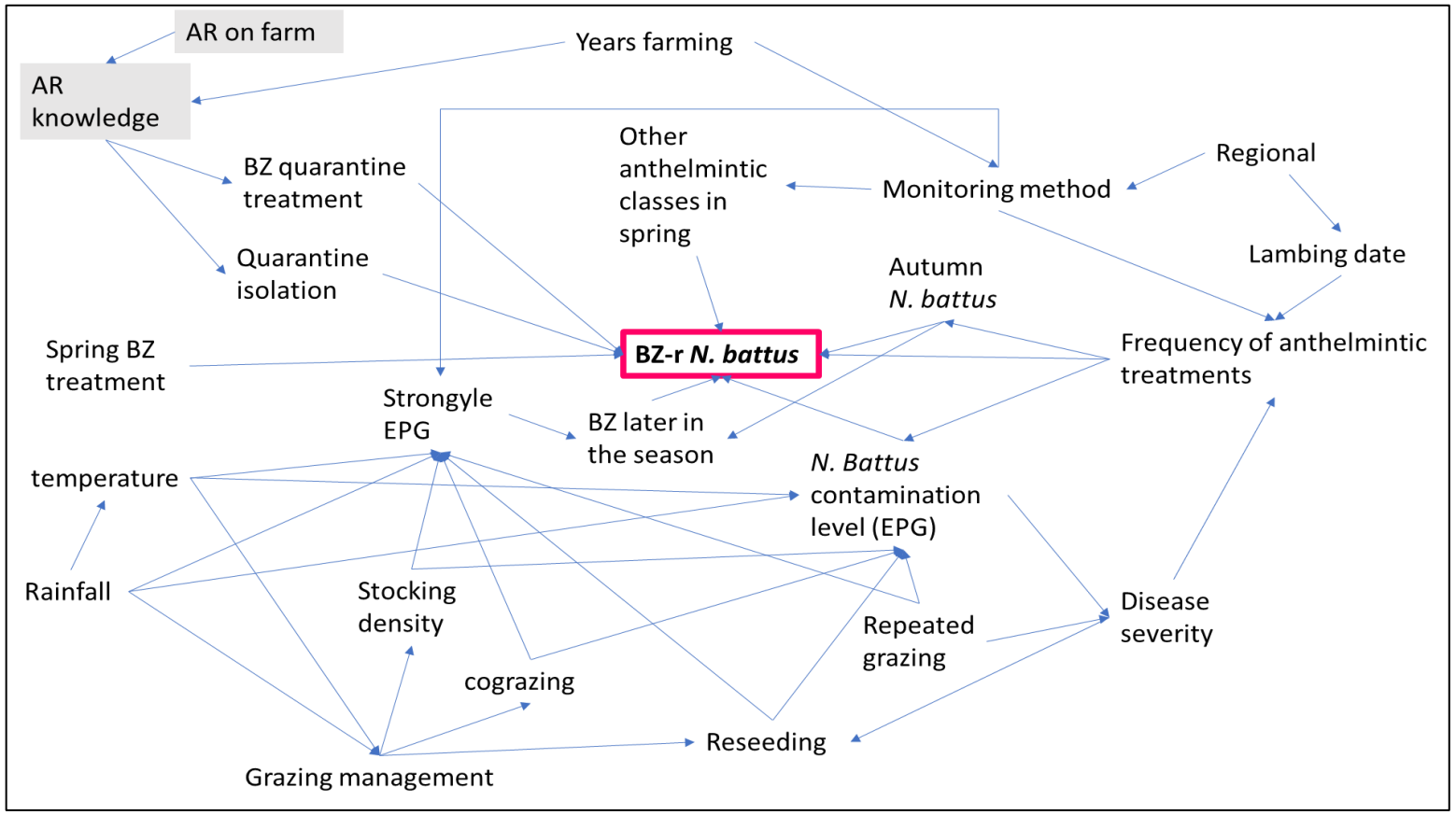


Figure 6.5. Causal web analysis examining the biological interaction between risk factors tested within the current GLMM model.

The arrows indicate potential biological links and the likely direction of influence. The grey boxes highlight additional factors which may have a significant influence on the system but for which data was not collected within the questionnaire described in chapter 5.

Causal web analysis was conducted based on biological reasoning to assess which factors may impact BZ resistance in *N. battus* and the interactions between them. Figure 6.5 highlights the complex interactions between environmental and management factors. Farmer knowledge about AR and AR diagnosed on farm in other trichostrongylid species, were highlighted during the exercise although data was not collected by the online questionnaire, indicating that the current analysis was not exhaustive and further factors may be implemented. The causal web was used to understand possible confounding effecting the building of fitted GLMM models. No further analysis was conducted using the causal web (e.g. directed acyclic graphs) due to the complexity of the web.

6.4.2.1 Forward inclusion model fitting

The forward inclusion GLMM method used resulted in the development of multiple models. The models were generated by adding the covariates in different orders. Different variables were retained in the final fitted models as the method adds variables in order, so each subsequent covariate is retained only if it explains a proportion of the variation not accounted for by previous factors. Thus, if two factors are confounded, only one would be retained within the multivariable model as both factors would explain the same proportion of the variation. The models were compared by the residual variation i.e. the model which described the greatest amount of the variation within the data (frequency of the F200Y resistant allele) was deemed to provide the most accurate description of the factors influencing resistance in this species.

Table 6.4. Model 1. Results of GLMM (fitted with a logit link) analysis with binomial outcome of the number of F200Y resistant alleles identified out of the total number of alleles identified on each farm. Results are presented as odds ratios, 95% confidence intervals and p-values (Wald tests). Analysis included information from 74 farms.

Fixed effect Coefficient		95% confidence intervals		
	Odds Ratio	Lower	Upper	P-value
SCOPS quarantine strategy (yes/no)	0.05	0.004	0.38	0.005
***N. battus* symptoms in spring only (yes/no)**	9.76	2.03	58.56	0.008
Intercept	0.003	0.0006	0.01	
Random effect Coefficient	Residual variance			
Farm ID	4.57			

Model 1 (Table 6.4) highlighted two factors which were significantly associated with the F200Y allele frequency at the 5% level. Resistant alleles were found to be over 9 times more likely on farms which observed *N. battus* symptoms primarily in spring (OR 9.8, 95% CI 2.0, 58.6). A negative association was identified with the use of quarantine strategies which follow SCOPS guidelines (OR 0.05, 95% CI 0.004, 0.4), indicating that resistant alleles are less likely to occur on farms implementing effective quarantine.

Table 6.5. Model 2. Results of GLMM (fitted with a logit link) analysis with binomial outcome of the number of F200Y resistant alleles identified out of the total number of alleles identified on each farm. Results are presented as odds ratios, 95% confidence intervals and p-values (Wald tests). Analysis included information from 61 farms.

Fixed effect		95% confidence intervals		
Coefficient	Odds Ratio	Lower	Upper	P-value
Vet plan for control of *N. battus* in lambs (yes/no)	6.00	1.06	35.38	0.033
Precipitation (mm)	1.04	1.02	1.07	>0.001
FEC monitoring for *N. battus* control in lambs (yes/no)	0.16	0.04	0.62	0.009
***N. battus* symptoms in spring only (yes/no)**	3.27	0.88	13.72	0.077
SCOPS quarantine strategy (yes/no)	0.03	0.003	0.17	>0.001
Supplementary feeding of lambs (yes/no)	0.34	0.17	0.59	>0.001
Intercept	0.01	0.0007	0.16	
Random effect				
Coefficient	Residual variance			
Farm ID	1.60			

Model 2 (Table 6.5) highlighted the use of effective quarantine strategy (following SCOPS guidelines) as having the greatest impact on resistant allele frequency in *N. battus*, reducing the likelihood of F200Y resistant alleles within the population by one third (OR 0.03, 95% CI 0.003, 0.17). The use of veterinary plans to determine parasite control was suggested to increase the odds of resistant alleles on farm (OR 5.99, 95%CI 1.06, 35.38). A negative association was also identified with monitoring faecal egg count to determine when to treat lambs (OR 0.16, 95% CI 0.04, 0.62). Supplementary feeding of lambs was identified as a potential barrier to resistance development (OR 0.34, 95% CI 0.17, 0.59), possibly

highlighting that animals maintained on a higher plane of nutrition require fewer anthelmintic treatments or perhaps graze less and therefore are subject to a lesser challenge. Annual mean precipitation on farm was also retained by the model with a small positive association with resistant allele frequency (OR 1.04, 95% CI 1.02, 1.07), although not directly linked to genotype frequency, precipitation level may impact on the viability and hatchability of the population and thus the fitness of the isolate.

Table 6.6. Model 3. Results of GLMM (fitted with a logit link) analysis with binomial outcome of the number of F200Y resistant alleles identified out of the total number of alleles identified on each farm. Results are presented as odds ratios, 95% confidence intervals and p-values (Wald tests). Analysis included information from 33 farms.

Fixed effect Coefficient	Odds Ratio	95% confidence intervals		P-value
		Lower	Upper	
***N. battus* symptoms in spring only (yes/no)**	0.81	0.08	7.85	0.853
Set stocking for lambs (yes/no)	149	17.71	1960	>0.001
Sporadic co-grazing of lambs with adult beef cattle (yes/no)	0.09	0.03	0.33	<0.001
Stocking density of lambs post weaning (per extra head, per acre)	0.58	0.42	0.76	<0.001
Reseeding of high risk pasture (yes/no)	6634	39.25	$2.0x10^6$	<0.001
FEC monitoring for GIN control in lambs (yes/no)	0.04	0.18	0.40	0.003
Intercept	3.95	1.00	8.17	
Random effect Coefficient	Residual variance			
Farm ID	1.09			

Model 3 (Table 6.6) retained several factors associated with grazing and grassland management. Strong, positive associations were observed with set stocked grazing of lambs and reseeding of fields routinely grazed by 1-3 month old lambs each spring which present a high *N. battus* risk to young stock (OR 149, 95% CI 17.71, 1960 and OR 6916, 95% CI 39.25, $2.0x10^6$ respectively). Sporadic co-grazing of lambs with adult beef cattle and stocking density of lambs post weaning were found to have a negative influence on the F200Y allele frequency of the population (OR 0.09, 95% CI 0.03, 0.33 and OR 0.58, 95% CI 0.42, 0.76 respectively).

6.4.2.2 Mixed approach model fitting

Models 2 and 3 retained the factor "*N. battus* symptoms in spring only" despite this factor not being statistically significant within the final models. Model 4 (Table 6.7) was the outcome of a backwards elimination of model 3, removing non-significant factors ($p>0.05$). Backwards elimination from model 2 resulted in retention of only one coefficient; the use of SCOPS quarantine strategy, the equivalent end-point as model 1.

Table 6.7. Model 4. Results of GLMM (fitted with a logit link) analysis with binomial outcome of the number of F200Y resistant alleles identified out of the total number of alleles identified on each farm. Results are presented as odds ratios, 95% confidence intervals and p-values (Wald tests).Analysis included information from 58 farms.

Fixed effect Coefficient	Odds Ratio	95% confidence intervals		P-value
		Lower	Upper	
Set stocking for lambs (yes/no)	7.44	1.88	33.78	0.004
Sporadic co-grazing of lambs with adult beef cattle (yes/no)	0.40	0.18	0.79	0.011
Intercept	0.01	0.003	0.03	
Random effect Coefficient	Residual variance			
Farm ID	3.32			

6.4.3 Model Selection

Models 2 and 3 were compared to model 1 by chi-squared analysis. Both models were found to explain the data better than model 1 (χ^2= 27.3 (d.f. =5), p<0.001 and χ^2= 12.5(4), p= 0.01 for comparison of model 1 with models 2 and 3 respectively). Models 2 and 3 could not be compared by chi-square as they used different data sets, comparison using a reduced data set shared by both models was not possible as model 2 failed to converge with the restricted data set. An F-test was used to compare the variance attributed to the random effect between models 2 and 3 (p= 0.15) therefore, both models explain a similar amount of the variance within the observed data. Model 4 was compared to models 1, 2 and 3 using an F-test and was found to explain a similar amount of the variation within the data in each case (p= 0.11, p= 0.996 and p= 0.998 when comparing model 4 to models 1, 2 and 3 respectively). Despite inclusion of a non-significant coefficient in the final model, model 2 was selected as it described a larger proportion of the variation observed than model 1. Selection of model 2 over models 3 and 4 was based on biological sense and because it incorporated a larger proportion of the data (models 2, 3 and 4 included data from 61, 33 and 58 farms respectively).

6.5 Discussion

The current study identified a range of factors positively and negatively associated with the presence of the resistant SNP mutation F200Y in UK *N. battus* populations. SCOPS recommended practices such as effective quarantine and the use of faecal egg counting to monitor the need for treatment were identified as protective factors against the development of BZ-resistance in *N. battus,* indicating that better awareness and monitoring of the problem on farm in conjunction with the timing, or number of treatment administrations may be important. Mixed species grazing and supplementary feeding were also associated with reduced resistant allele frequency whilst reseeding contaminated fields and set stocked grazing were identified as potential risk factors.

The hypothesis that the development of BZ-resistance in *N. battus* was similar to that of other strongyle species i.e. influenced by the frequency of anthelmintic treatment and quarantine practices was partially correct. From the results of the present study, quarantine appears to play a significant role in determining the presence of F200Y resistant alleles within the population. Key risk factors highlighted as being selective for resistance development in other strongyle nematodes infecting sheep and cattle include; the frequency and timing of anthelmintic treatment and movement of animals onto low contamination fields immediately post-treatment (dose and move) (Falzon et al., 2014; Hughes et al., 2007; Lawrence et al., 2006; Suarez and Cristel, 2014; Suter et al., 2004; Vadlejch et al., 2014), which were not selected by the current analysis.

A total of four models were developed during this study. Models 1-3 were achieved by adding fixed effect factors in different orders. Model one focused on the importance of quarantine, the second was based on monitoring strategies and nutrition and the third was based on grazing management. The analysis method used excluded data points which were missing a response for any of the fixed effects included in the model, namely supplementary feeding,

reseeding and typical stocking densities and were influenced by the order in which coefficients were added to the model. Survey questions were not mandatory, participants were free to skip questions which they felt did not apply to their farming enterprise and so question responses had different numbers of data points. Models two and three were therefore fitted to different data sub-sets, representing different farming cohorts. Statistical comparison found that model two explained the data set best however, this was likely influenced by the increased number of questionnaire responses included in this model (n=61), compared to model three (n=33). Both models provided a good fit for their respective data sub-sets. Models 2 and 3 both retained a non-significant factor within the final model "*N. battus* symptoms observed in spring only". Inclusion of a non-significant coefficient within the final model was an artefact of the forward inclusion method used to build the models i.e. the factor was significant when added and retained within the model but became non-significant following the addition of other covariates. Further exploration was performed, working through a backwards elimination process from models 2 and 3; a mixed approach. The resultant output (model 4) retained only set stocked grazing of lambs and sporadic co-grazing of lambs with adult beef cattle as factors potentially increasing and decreasing the risk of identifying the F200Y resistant allele on farm respectively. From statistical comparisons, models 2 and 3 explained a greater proportion of the variance observed in the data than the simpler models 1 and 4. The data set upon which the models were based and thus the outcomes were influenced by the order in which coefficients were added during model development. However, as there has been no previous research into anthelmintic resistance risk factors for this species, it was difficult to determine which factors to begin with and therefore which fitted model was the best. The findings from all models have therefore been discussed within this chapter as they highlight management practices which may be important in different farming systems.

The number of farms available for inclusion in the current analysis which had >5% resistant alleles in their *N. battus* population and matching management questionnaire data was low. Given the relatively low number of farms and the high number of variables tested, the chance of correlation and confounding within the GLMM analysis was high. Causal web analysis (Figure 6.5) was conducted to assess the biological sense of interactions between factors, how these could potentially impact the development of BZ-resistance in *N. battus* and to examine causality between the factors included in the GLMM analysis. Although the web is not an exhaustive list of the factors which could possibly influence this parasite population within the field, the complexity of the system is evident from the number of interactions within the web. Although the chance of correlation is high, the complex interactions make it almost impossible to rule out factors which were highlighted by the analysis. Many covariates were tested during univariate analysis, around 5% could be found to be significant by chance (type 1 error). One possible example may be the specific use of Albex™ (Chanelle, UK), a commercial product containing albendazole, found to be correlated with F200Y allele frequency during initial univariate analysis but not retained in any of the fitted multivariate models. It is possible that this result may have been true or may have been confounded with another aspect of farm management however, extensive experimental validation would be required to prove or disprove the influence of this or any other factor. The findings discussed in this chapter can therefore be interpreted as indicators of the direction of future study in this area.

The presence of *N. battus* throughout the grazing season could have allowed for additional selection pressure from frequent anthelmintic treatments aimed at strongyle nematode species perhaps explaining the strong, positive association between set stocked grazing and resistant allele frequency. However, each treatment would impact only a proportion of the population, unlike treatments administered to spring-hatching populations therefore reducing selection pressure on the population overall. Preliminary screening analysis

identified that high resistant allele frequency was strongly associated with farmers' perceptions of acute spring infection rather than low level infection throughout the grazing season. The association of resistance and spring infection may indicate a role for parasite refugia in AR development in *N. battus*. Whilst the maintenance of refugia has been identified as an important factor in delaying the development of AR in trichostrongylid species (van Wyk, 2001), the impact on *N. battus* is difficult to assess given the differences in life history traits between the species.

Reseeding of contaminated fields was found to be a potential risk factor for the development of resistance in *N. battus* during the current analysis. This practice dramatically reduces parasite contamination, providing a severe population bottleneck and places great importance on the re-infection of 'clean' grazing to establish a population of mixed genotypes for the following year. The risk posed by re-infection of reseeded pasture is similar to that represented by dose and move strategies as the clean grazing contains few parasites available to dilute any resistant individuals surviving treatment (Falzon et al., 2014; Hughes et al., 2007; Waghorn et al., 2009; Waller et al., 1989). Reseeding also increases forage quality however, anecdotal accounts suggest that grazing lush, fresh ley grass can result in diarrhoea in lambs due to over-eating and the high water content of the grass. Increased digesta flow rate has been associated with alteration of the metabolism and absorption of anthelmintics (Taylor et al., 1992), potentially resulting in a sub-optimal dose being retained within the animals which may allow survival of heterozygote resistant parasites.

Mixed species grazing was highlighted as significantly reducing the odds of identifying the F200Y resistant allele on farm. The influence of alternate or co-grazing of sheep and cattle on the parasite population is difficult to quantify and will differ dependent upon the nematode species of interest and its co-infectivity between ruminant species (Morley and Donald, 1980). The results of previous studies on AR in GIN were varied (Eddi et al., 1996;

Lawrence et al., 2006) suggesting that co-grazing has the potential to enhance or reduce resistance development in different farming systems. Co-grazing has been associated with an increased risk of AR in GIN due to a reduction of the number of parasites in refugia (Good et al., 2006) however, *N. battus* is known to successfully infect calves (Bairden and Armour, 1987; Coop et al., 1991; Coop et al., 1988; Coop et al., 1984), and although larvae will be removed from pasture through grazing, the calves will still contribute to the population on pasture. Additionally, they are unlikely to be treated at the same time as the sheep, thereby maintaining genes for susceptibility in the population. Reduced exposure of lambs to GIN may trigger fewer treatments given less severe clinical symptoms, reducing the selection pressure on the population. Less severe symptoms could also safeguard against sub-optimal dose retention of any anthelmintic treatments administered. Ewe grazing also has a major impact on pasture contamination and subsequent exposure of lambs. During univariable analysis, the odds of F200Y alleles in *N. battus* was reduced with increasing number of anthelmintic treatments administered to ewes per year. The use of ill thrift monitoring to determine the timing of treatment was also found to have a significant effect, reducing the odds of AR in *N. battus* by 90%. The use of ill thrift monitoring in ewes may reduce the number of treatments administered, particularly around lambing and mating, potential maximising *N. battus* refugia in spring, reducing the selection pressure from BZ treatment of lambs. However, the association between the number of treatments given to ewes and resistance appears counter-intuitive. Fewer ewe treatments may increase pasture contamination, particularly in spring, potentially leading to a greater parasite challenge for the lambs which may trigger more frequent treatment, thus increasing selection pressure.

Cattle grazing also changes pasture characteristics; reducing sward height, altering clover composition and nutritional content of forage crops (Nolan et al., 2001). Studies of pasture following different grazing regimes found that forage quality was significantly greater on land grazed by sheep and cattle compared to sheep-only grazing (Abaye et al., 1994). Cattle graze

non-discriminately which restricts ryegrass, allowing for the expansion of protein rich species such as white clover (Nolan et al., 2001). Several authors have noted lamb performance benefits given mixed species grazing and increased white clover content of pasture (Abaye et al., 1994; Niezen et al., 2002a) suggesting that the influence of co-grazing on parasite management may result from increased nutrition. Nutrition, in the form of supplementary feeding, was again highlighted as a protective factor with supplementary fed lambs less likely to harbour *N. battus* carrying the BZ-resistant SNP F200Y. Increased protein intake has been shown to increase the resilience of sheep to parasitic infection (Coop and Kyriazakis, 1999, 2001), reducing the requirement for anthelmintic treatment and therefore the selection pressure on the population. Studies on resistance to the macrocyclic lactones (3-ML) identified narrow wool fibre diameter as a risk factor (Lawrence et al., 2006), the authors suggested that this may represent breed differences however, the nutritional plane of the animal may also be implicated. Increased crude protein intake has been shown to increase wool production and fibre diameter (van Houtert et al., 1995). Increased worm burden either as a result of, or leading to lower nutritional status may trigger more frequent anthelmintic treatment, subsequently increasing the selection pressure for AR. Supplementary feeding could also have an indirect influence, feeding may reduce grazing which in turn reduces exposure to parasites. Lower exposure of lambs to GIN would reduce diarrhoea in the flock, triggering fewer anthelmintic treatments and ensuring optimal metabolism and absorption of treatments administered.

Alteration of the dietary forage has also been shown to influence parasite egg development and subsequent hatching proportions, possibly due to variation in the concentration of condensed tannins within the faecal matter or moisture content (Niezen et al., 2002b). Shorter sward heights following cattle grazing may also result in increased exposure of larvae to UV light reducing their longevity on pasture (van Dijk et al., 2009). High stocking density of lambs post weaning could have a similar impact on sward height and larval survival if

pasture is eaten down late in the season without sufficient time to recover prior to *N. battus* hatching.

Set stocked grazing of lambs was highlighted as a significant driver of BZ-resistance in the current analysis. Perhaps due to the repeated exposure of the nematode population to treatments throughout the grazing season due to the constant availability of hosts for infection. Alternatively, the use of set stocked grazing may increase the reliance on anthelmintics to control parasite infection, potentially increasing selection pressure for the nematode populations.

Environmental factors have a strong influence on the hatching of nematode eggs and subsequent exposure of lambs to larvae on pasture. Several environmental factors were included during model development to explore their possible associations with the development of resistance in *N. battus*. With the exception of precipitation, environmental factors were not found to influence the development of BZ-resistance in *N. battus*. The link with rainfall could be confounded with other variables such as geographic location; for example North West England was identified as a focal region of resistance in this species (chapter 2), which is a high rainfall region. Previous research identified a potential link between cool, wet conditions and increased prevalence of BZ-resistance in Spain however, no direct impact of moisture availability was identified (Calvete et al., 2012). Moisture level was deemed to have a minimal influence on *N. battus* egg hatching (Parkin, 1975; van Dijk and Morgan, 2012). Previous reports have suggested that a sharp increase in moisture level, following prolonged dry weather, could act as a hatching stimulus in the absence of the normal chill stimulus (Gibson and Everett, 1981; Rickard et al., 1989) however, rainfall or moisture level is intrinsically confounded with temperature and UV level therefore likely to impact on hatching and larval survival at pasture via other pathways as demonstrated in Figure 6.5. High rainfall could also accelerate faecal breakdown on pasture, *N. battus* eggs

have been shown not to develop within faecal pellets (van Dijk and Morgan, 2008), eggs washed out of faeces into soil develop quicker with lower mortality rates (Gibson and Everett, 1981). It is feasible therefore to hypothesise that high precipitation levels may benefit parasite survival and viability or influence the timing or magnitude of larval availability, increasing the challenge to lambs and potentially the frequency of anthelmintic treatment and thus, increasing the selection pressure for resistance.

As well as geographic trends in environmental factors, chapter 5 identified significant regional variation in the monitoring practices used to determine the need for treatment highlighting greater use of evidence-based drenching in the South. Lower BZ-resistant allele frequency was associated with the use of FEC in the control of *N. battus* infection in lambs. Although not generally advised for *N. battus* during acute spring infection, FEC monitoring could delay or reduce the number of treatments administered. Waiting until eggs are detected in faeces could result in eggs being deposited onto pasture prior to treatment, increasing the population of unselected parasites on pasture to dilute any surviving treatment for the following spring. Delaying treatment could help protect anthelmintic susceptibility but could also result in production losses from delayed intervention.

The use of FEC to monitor *N. battus* may also indicate a shift in the timing of infection in recent years, from predictable spring incidence to low level infection throughout the grazing season. Gethings *et al.* (2015) predicted that lambs grazing in the South may miss the peak *N. battus* challenge as the timing of egg hatching shifts earlier in the season due to climatic warming. The misalignment of lamb grazing and the peak *N. battus* larval challenge could result in low level infection, likely triggering fewer anthelmintic treatments at different times. The timing of anthelmintic treatment has been repeatedly highlighted as a risk factor for AR development in other GIN species, particularly treatments administered in autumn and winter when parasite population on pasture is naturally low (Suarez and Cristel, 2014;

Suter et al., 2004; Vadlejch et al., 2014). The use of FEC to inform the control of other GIN species in lambs throughout the year was also highlighted by the analysis. Despite the association of FEC usage and increased anthelmintic treatment frequency in chapter 5, the practice was suggested to reduce the odds of anthelmintic resistance in *N. battus*. The number of anthelmintic treatments was collected overall, throughout the season therefore not specifically aimed at *N. battus* control. Although a direct influence on *N. battus* populations is not clear, implementation of such recommended practices and detailed knowledge of the parasite population on farm have been linked with the adoption of further 'best practice' methods and advice (Jack et al., 2017). Conducting frequent FECs on farm will build a picture of the timing of infection and contamination levels of different fields throughout the farm which may influence grazing decisions and management on farm. Post treatment FECs are likely to be more commonly used by farmers who use FEC as a routine diagnostic, reducing the use of ineffective drugs, potentially minimising production losses and further advancement of resistance against that anthelmintic class.

Greater awareness and knowledge of AR has been highlighted as a driver for the uptake of 'best practice' recommendations (Jack et al., 2017) and so may influence other management decisions on farm. The strong association between resistant allele frequency and set stocked grazing may be confounded with the likelihood to adopt SCOPS recommended practises, supported by the fact that these factors did not appear within the same multivariable model. Chapter 5 found that set stocked grazing was more commonly used by respondents who had been earning a living from farming at their current address for over 20 years. A recent study about farmer attitudes and uptake of novel recommendations found a strong link between farmer age and uptake/knowledge of SCOPS guideline practices aimed at slowing AR development, suggesting poor adoption by older farmers (Jack et al., 2017).

Contrary to the association of recommended practice with reduced resistant allele frequency, the use of veterinary parasite control plans to determine treatment timing for *N. battus* control was significantly linked with high F200Y frequency. Veterinary plans are typically designed using detailed farm information such as drug efficacies together with the parasite and grazing history of fields. It is unlikely that the use of a veterinary plan to determine anthelmintic treatments would increase the odds of resistance developing so it can be presumed that the association observed in the model represents farms with known or suspected resistance problems seeking advice and assistance with future treatment decisions.

As discussed in chapter 5, the increasing prevalence of AR on UK farms, SCOPS developed a set of quarantine guidelines to assist farmers in effectively minimising the introduction of resistant parasites onto farm by new and returning stock. The guidelines advise that all incoming stock should be treated using at least one of the new anthelmintic compounds (4-AD or 5-SI) and be kept separate from existing stock for a minimum of 21 days (Abbott et al., 2012). Our questionnaire survey identified that around half of respondents routinely employed quarantine strategy of any kind and only 10% followed the SCOPS guideline practices, similar to previous findings (Morgan et al., 2012). The current analysis identified that following the SCOPS guidelines on quarantine was a barrier to the development of BZ-resistance in *N. battus*. The significance of quarantine treatment and isolation within the analysis suggesting that animal movement is likely to be an important factor in the spread of AR in this species. This is in agreement with previous studies which identified the administration of quarantine treatments as a barrier to the establishment of AR in strongyle species (Suter et al., 2004; Vadlejch et al., 2014) and found a significant correlation between the numbers of sheep brought onto farm each year and AR frequency (Lawrence et al., 2006). Despite the high volume of sheep movement in the UK, trade of young lambs during the peak *N. battus* period is minimal. The movement of older animals in spring may therefore be more

important than the movement of lambs however, information was not collected on the movement of older animals in the present study.

6.6 Conclusion

Results of the current analysis highlight the use of recommended practices, designed to slow the dissemination of anthelmintic resistance, nutrition level of the host and factors which significantly reduce the parasite population on pasture as important impacts on the emergence and dissemination of resistance in *N. battus*. Despite significant differences in the epidemiology of *N. battus* compared to other GIN species, some of the factors which have been previously linked with the development of anthelmintic resistance in strongyle nematodes were also highlighted by the current models, suggesting that certain recommendations for slowing the development of AR in GIN may also be applicable to *N. battus*. Minimising the risk of resistance in spring populations may be challenging given the high pathogenicity of *N. battus* therefore, implementation of effective quarantine may be the most effective control strategy given the results of the current study.

7 *Nematodirus battus* egg hatching: Investigating the drivers of change

7.1 Abstract

Since the Nineties, it has been proposed that the seasonality of *Nematodirus battus* may be changing from strictly spring towards additional autumn infection. Over the past decades, anecdotal evidence has continued to suggest that this is the case and hypotheses for drivers have been proposed. However, the likely effect of drivers, such as changing climatic and farm management factors, and their relative importance, has not been quantified. Historically, eggs were described as requiring a period of chilling over winter for synchronous hatching to occur when climatic conditions became optimal, in spring. Egg hatching without prior chilling has been demonstrated *in vitro* and observation of *N. battus* infection throughout the year may indicate that a proportion of eggs indeed no longer require chilling before hatching. Changes in the timing of parasite challenge can result in unexpected disease outbreaks. It is therefore pertinent to understand what drives changes in hatching behaviour, with a view to informing effective control strategies, minimising production losses and associated economic costs. The present study investigated the requirement for chilling in 90 UK *N. battus* populations from commercial sheep farms and potential environmental and management factors which may influence hatching *in vitro*. Between 0 and 87% of eggs were able to hatch in the absence of a chill stimulus (non-chill hatching) (mean %±SEM; 13±1.7). Variation between populations was high, with a geographic trend towards higher non-chill hatching in the North. Generalised linear mixed modelling (GLMM) identified associations between spring temperature, the timing of lambing and several grazing management strategies with non-chill hatching. The results indicate a possible role for management factors such as resting and reseeding of fields routinely grazed by young lambs, potentially interrupting the *N. battus* lifecycle.

7.2 Introduction

The hatching behaviour of *Nematodirus battus* populations appears to be changing. This highly seasonal parasite is typically responsible for acute disease in lambs in late spring (Boag and Thomas, 1975; Thomas, 1990). In recent years however, reports of *nematodirosis* in late summer and autumn have increased (Sargison et al., 2012).

N. battus has a direct parasitic lifecycle but, as opposed to other gastrointestinal nematodes, the infective larvae (L_3) develop within the egg rather than on pasture. Developed L_3 typically remain encased within the egg throughout the winter/cool months. Once environmental conditions become optimal for larval survival; mean day and night temperature greater than 10°C for 10 days [1], synchronous hatching of eggs occurs, resulting in large numbers of infectious larvae on pasture [2]. The winter/chill stimulus was originally believed to be essential to *N. battus* hatching (Thomas and Stevens, 1960) but more recent work has demonstrated large proportions of egg populations hatching without chilling (van Dijk and Morgan, 2008; van Dijk and Morgan, 2010) however, this has not been quantified using field populations. The precise role that non-chill hatching, observed in temperate regions, plays in the epidemiology and biology of the parasite remains unclear. Chilling stimulates the encased larvae to transform lipid energy reserves to trehalose sugar which lowers the temperature at which larvae freeze; protecting the parasite over winter (Ash and Atkinson, 1983) and has also been linked to increased longevity post hatching, compared to larvae hatched without prior chilling (van Dijk and Morgan, 2008). *N. battus* is believed to be a species of arctic origin (Hoberg, 2005; van Dijk and Morgan, 2008), for which increased cold hardiness and longevity on pasture could be important adaptations, increasing the likelihood of survival during periods of host absence. On modern, intensive, sheep farms, however, hosts are plentiful year-round, and larval longevity on pasture may not be as crucial. Hatching

without chilling would increase transmission opportunities and could therefore be more beneficial to the parasite in the UK.

Although *N. battus* has been described as a spring disease for the past 50 years, autumn hatching was documented as far back as 1960, albeit at very low levels (Thomas and Stevens, 1960). Further reports were published in later years (Boag and Thomas, 1975; Gibson and Everett, 1981; Hollands, 1984; Rodgers, 1983; Thomas, 1990) and recently *N. battus* has been reported to result in clinical cases in autumn (Sargison et al., 2012). The drivers of non-chill hatching and autumn *nematodirosis* have not been quantified however, it is possible that changes in farming practice, such as avoiding repeated lamb grazing of heavily infected fields each spring may have selected for non-chill hatching as a means of increasing possible transmission opportunities on some farms (van Dijk and Morgan, 2008; van Dijk and Morgan, 2010).

Optimal conditions for chilled *N. battus* egg hatching have been previously studied (Figure 7.1); identifying temperature as the key driver; hatching is restricted to ambient temperatures within the range of 11-17°C (van Dijk and Morgan, 2008), with other environmental factors such as moisture not believed to provide hatching stimuli (van Dijk and Morgan, 2012).

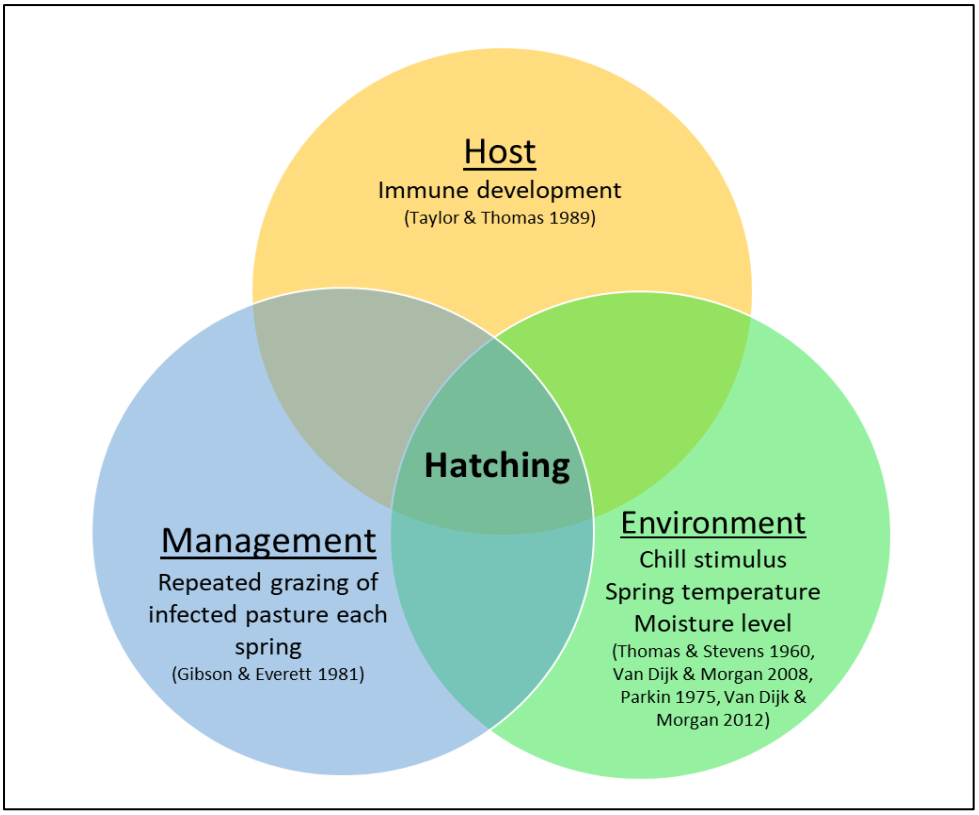


Figure 7.1. Venn diagram of factors currently believed to impact on the hatching of N. battus eggs following a chill stimulus from literature.

Previous descriptions of non-chill hatching found high levels of variation between farms (van Dijk and Morgan, 2008; van Dijk and Morgan, 2010) and although hypotheses relating to temperature (van Dijk and Morgan, 2008; van Dijk and Morgan, 2010), avoidance of co-infection with competing worm species (van Dijk and Morgan, 2010) and host availability (Gethings et al., 2015) have been proposed, the influence and relative importance of proposed drivers has not been quantified. Knowledge of the drivers of non-chill hatching and the extent of this phenomenon appears pertinent for the design of improved control methods.

The aim of the current study was to quantify the proportion of eggs able to hatch in the absence of a chill stimulus in a large number of UK *N. battus* populations, to explore the variation in hatching behaviour at the individual farm and regional level, to examine the

possible link between the ability of eggs to hatch without a chill stimulus and the presence of the primary SNP associated with BZ-resistance in *N. battus* (F200Y) and to investigate the association between chilling requirement of eggs and potential drivers of non-chill hatching. The likely influence of environmental factors and farm management strategies on hatching of eggs with and without a prior chill stimulus was analysed and a predictive model constructed to test if farm management or host factors were stronger determinants of *N. battus* hatching behaviour than climatic factors.

7.3 Methods

7.3.1 Sample collection

A subset of the *N. battus* populations described in chapter 2; 90 populations from 73 farms collected between June and August in 2015 and 2016, were included in the hatching experiment (Figure 7.2). Populations originating from the same farm were collected at different time points or from fields with different grazing histories. Sampling was restricted to spring/summer in order to standardise the likely origin of eggs, i.e. these eggs are likely to have originated from adult worms which developed after infection with spring-hatched larvae. A total of 10 populations were submitted by Scotland's Rural College (SRUC) surveillance centres in Ayr and Dumfries. Samples were collected opportunistically in 2015 in a non-random fashion (n=61). Sampling in 2016 (n=19) was targeted to regions which were under-represented by the *N. battus* sample biobank collected in 2015 but which appeared to have significant sheep density (sheep density data from the Office for National Statistics in 2009 was mapped using QGIS (Las Palmas version 2.18); data source Geo-wiki). Farms in the target regions were contacted via local advisors, veterinarians and the Animal and Horticulture Development Board (AHDB).

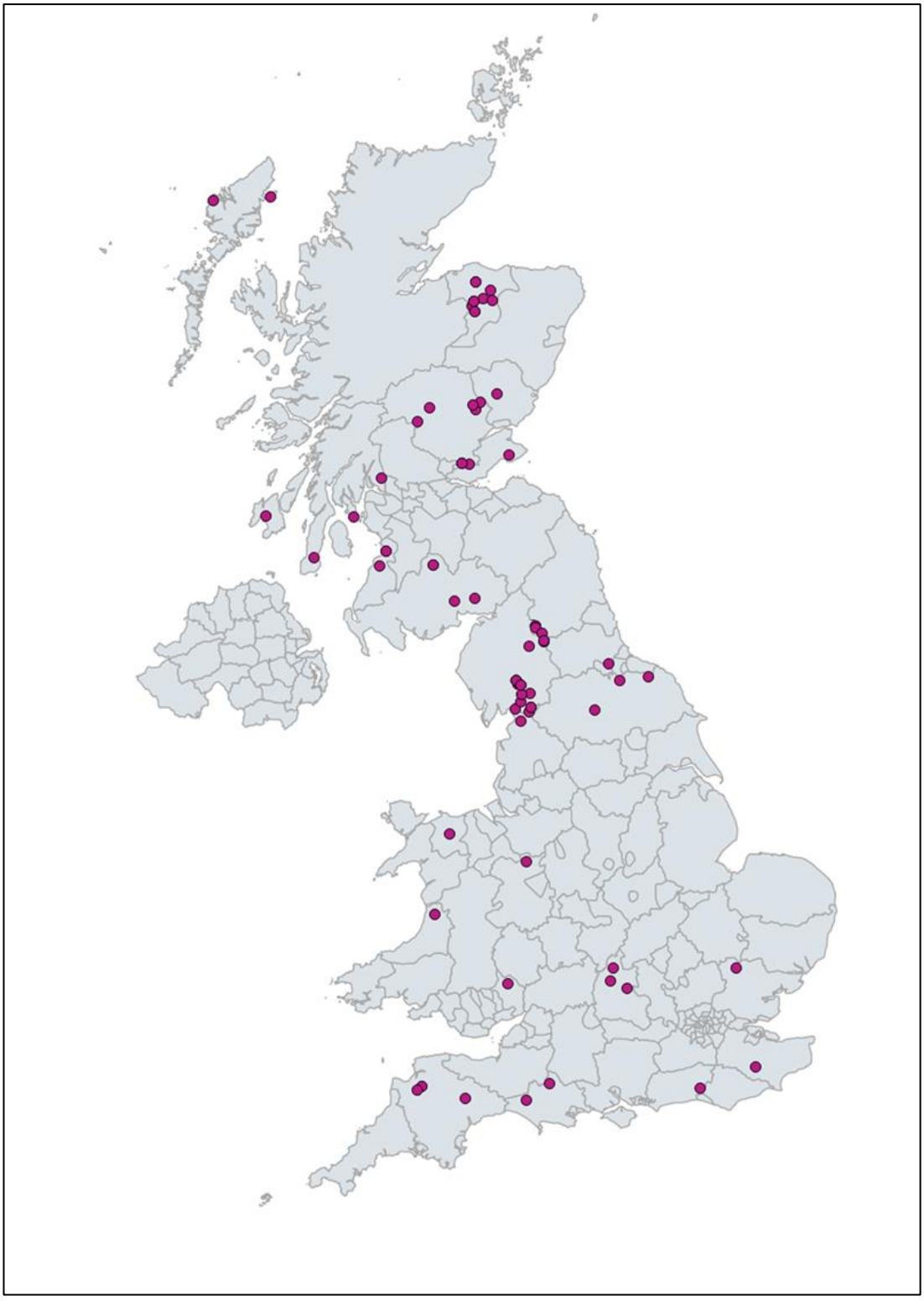


Figure 7.2. Map of the origins of N. battus populations included in the hatching study.

Samples were collected as described in chapter 2. Faecal samples were collected in plastic bags with excess air removed and stored at 4°C once returned to the laboratory to suspend development prior to processing. Samples which were submitted by SRUC surveillance centres or posted to the laboratory directly from farmers were packaged in air-tight containers for postage and stored at 4°C upon arrival, prior to processing. The impact of chilling prior to development was deemed minimal as known physiological changes as a result of chilling are believed to occur within the developed larvae, rather than the undeveloped egg (Ash and Atkinson, 1983).

7.3.2 Sample preparation

Faecal egg counts were conducted on all samples to confirm the presence of *N. battus* eggs prior to egg extraction (Jackson and Christie, 1972).

Eggs were extracted from the faeces by differential sieving as described in chapter 2. Once extracted, eggs were placed in non-air-tight jars with tap water. Egg cultures were stored at ambient room temperature, protected from direct sunlight. Cultures were monitored microscopically for development of third stage larvae within the egg shell (Figure 7.3). Previous work by van Dijk and Morgan (2008) observed maximal development of *N. battus* eggs to this stage in 44-52 days at temperatures between 15 and 20˚C.

Figure 7.3. N. battus egg at the embryonated stage, L_3 visible inside the egg.

7.3.3 Hatching experiments

7.3.3.1 'Chill' Incubation

Three aliquots of approximately 500 developed *N. battus* eggs per population were transferred to 6 well cluster plates (Sarstedt, Germany) in 7ml tap water. Eggs were placed at 4°C (range 2-8˚C) for 6 weeks then incubated at the optimum hatching temperature for *N. battus*; 13°C (range 13-15˚C) for a further 4-6 weeks. Incubation temperature of 13°C was chosen in this study based on the findings of previous work which indicated that 13°C provided the strongest hatching stimulus for isolates from Edinburgh and Bristol (van Dijk and Morgan, 2010).

7.3.3.2 'Non-chill' Incubation

A further three aliquots of ~500 embryonated eggs were transferred to 6-well cluster plates as above and incubated at 13°C (range 13-15˚C) for 10 weeks without prior chilling.

Following incubation, suspensions were thoroughly mixed and 1.4ml was removed from each well, transferred to a 24 well plate and fixed with helminthological iodine for counting. The number of third stage larvae and developed eggs were recorded for each well.

7.3.4 Model construction

7.3.4.1 Data collection

Climatic data for risk factor analysis was obtained and the values for each farm location were extracted as described in chapter 6. Farm management data was collected by online questionnaire as described in chapter 5. Table 6.1 contains a summary of the covariates tested and how each factor was coded.

7.3.4.2 Data Analysis

7.3.4.2.1 Binary logistic regression

Each hatching experiment was conducted in triplicate, comparison of these technical replicates was conducted using binary logistic regression analysis. Binary logistic regression was also used to compare the magnitude of hatch between UK countries during initial descriptive statistical analysis and to assess the overall impact of chilling. In all GLM analyses, the outcome response tested was whether eggs had hatched or not, each factor (chill, country or technical replicate) was included as a fixed effect. As the typical hatching conditions of *N. battus* include a chill stimulus, chilling was coded as 0 within the models and non-chill was coded as 1.

Linear regression analysis was used to explore the possible correlation between the proportion of eggs hatched per isolate, with and without a period of chilling, and the F200Y resistant allele frequency of the population using data generated in chapter 2. Analyses were carried out using R (version 3.2.5). Statistical significance was defined at the 5% level (i.e. $p<0.05$) for all analyses.

7.3.4.2.2 Modelling

Generalised linear mixed modelling (GLMM) was employed to investigate the impact of combinations of environmental and management factors on the hatching patterns observed during *in vitro* experiments. Briefly, for each 'chilling' treatment, the outcome variable was the number of worm eggs hatching, compared to the number tested: a binomial model. No lower threshold for number of eggs was used therefore, all populations were included in model development as the GLMM method accounts for differences in sample size (i.e. number of eggs in each population) and weights the influence on the model outcome of each population appropriately. Covariates considered were: whether or not a chill was applied to the eggs, farm demographic, farm management and climatic factors listed in table 6.1.

Sample storage times (time in faeces: between collection and egg extraction, egg development: between extraction and experimental set up) were forced into the models to account for variation in experimental set up between populations. Farm was fitted as a random effect to account for clustering at farm level. Multiple analyses were conducted in this study to allow for analysis of management, environmental and host coefficients. Many of the factors tested were correlated, such as weather conditions with geographic location, the GLMM method used accounted for confounding as each factor is added in order, therefore the estimate and significance value reported represents the amount of variance explained by that factor after the impact of all previous factors have been accounted for by the model. Due to the lack of previous work on the drivers of hatching in *N. battus*, investigation of all possible factors was important. Model 1 examined associations between environmental factors and the proportion of *N. battus* eggs hatching with and without a chill stimulus. Model 2 focused on the impact of farm management strategies and the third model investigated the influence of lambing date and how this varied between farm types. Models were built and tested using R (version 3.2.5). A mixed model development approach was used: a combination of forward inclusion and backward elimination, to generate a parsimonious model that was both biologically plausible and contained only covariates that were statistically significant at the 5% level (i.e. p-value < 0.05 in the fixed effect summary). Fitted models were compared using a likelihood ratio test to determine the best fit for the data.

7.4 Results

The average faecal egg count of samples submitted was 161 *N. battus* eggs per gram (EPG, range 1 - 3330), with average counts from individual farms ranging from 1 to 1250 EPG. Prior to faecal egg count and extraction eggs were stored in faecal matter at 4°C for between 1 and 33 days (mean 11, median 9). At the point of faecal egg counting, eggs appeared morphologically un-developed; only morula were observed, and no early-stage larvae within the egg shells. Once extracted, eggs were incubated at ambient room temperature for an average of 46 days (range 15-98 days, median 42 days) to allow for larval development.

An average of 121 eggs/larvae were counted per replicate (range 9-540). Each hatching experiment was conducted in triplicate, which is each sample was divided into six subsamples and three were observed for hatching following a chill stimulus, and three without. In order to test that there was no systematic biases in the protocol, differences between technical replicates was checked for in the model. No such biases were found ($p=0.4$). Only two isolates were included from Wales as few samples with sufficient eggs for inclusion in the hatching experiment were collected from this region, results from these isolates were combined with those collected from England for analysis; England and Wales n=50, Scotland n=40.

The proportion of the total number of eggs in suspension which hatched under experimental conditions varied greatly between farm populations. The influence of the chill stimulus was assessed by binomial logistic regression analysis, and concluded that the absence of chilling (non-chill) dramatically reduced the proportion of eggs hatched in the majority of populations, regardless of origin (OR 0.13, 95% CI 0.12, 0.13, $p<2x10^{-16}$). The median proportion of eggs hatched with and without chilling was 0.453 and 0.0420 respectively. However, non-chill hatching was greater than chilled hatching in seven populations tested, these populations were not aggregated geographically.

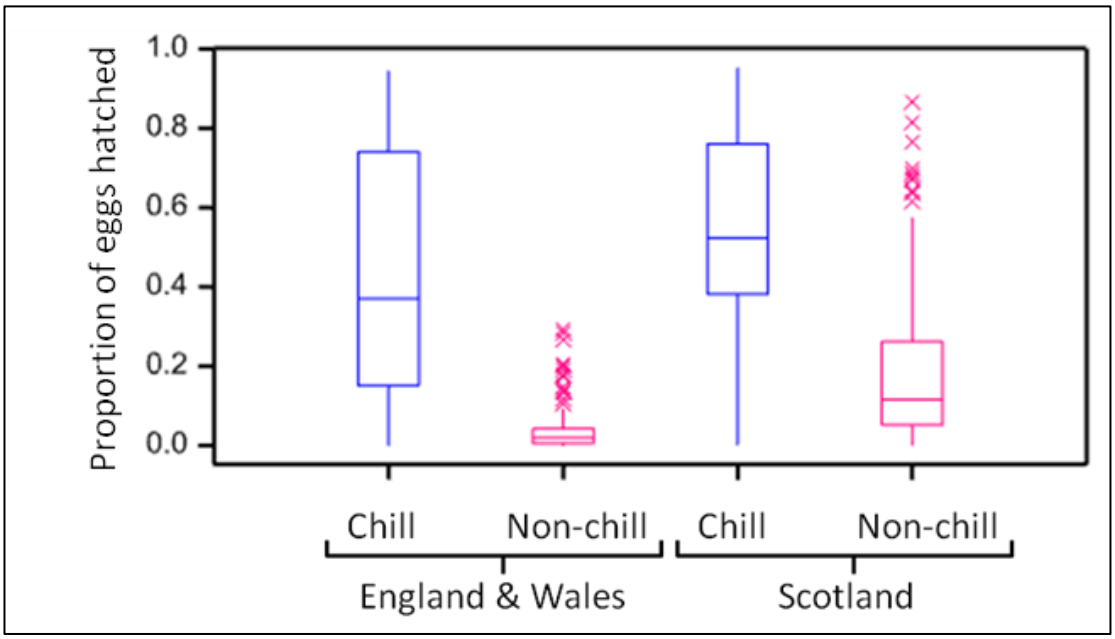


Figure 7.4. Summary of chill requirement of eggs from Scotland and England/Wales.

Proportion of eggs hatched with (blue) and without (pink) undergoing a period of chilling organised by country of origin.

The trend of higher hatching in the North was observed both with and without chilling (Figure 7.4). The mean hatch rate with chilling was (mean%±SEM) 56%±2.4 in Scotland (range 0 –95) and 41%±2.4 for England and Wales (range 0–95). Hatching without chilling was around 5.5 times higher in Scottish isolates compared to the rest of the UK; 22%±2 (range 0–87) compared to 4%±0.5 (range 0–35). Binary logistic regression analysis found the difference in proportional hatch without chilling to be statistically significant at the 5% level between the countries (OR 2.03, 95% CIs 1.97, 2.12, $p<0.0001$) i.e. the odds indicate that eggs obtained from populations in Scotland were twice as likely to hatch without a prior chill stimulus.

7.4.1 Comparison of F200Y resistant allele frequency and altered hatching requirements in *N. battus* populations

Figure 7.5 shows the correlation of F200Y resistant allele frequency (chapter 2) and the proportion of eggs hatched with and without a chill stimulus. Regression analysis found no statistically significant associations between hatching and resistant allele frequency with or without a chill stimulus at the 5% level (OR 0.99, 95% CI 0.85, 1.16, p=0.92 and OR 0.87, 95% CI 0.67, 1.12, p=0.27 for chill and non-chill hatching respectively) suggesting that variation in the hatching behaviour of *N. battus* is not likely to be a significant driver of the emergence of BZ-resistance in the species.

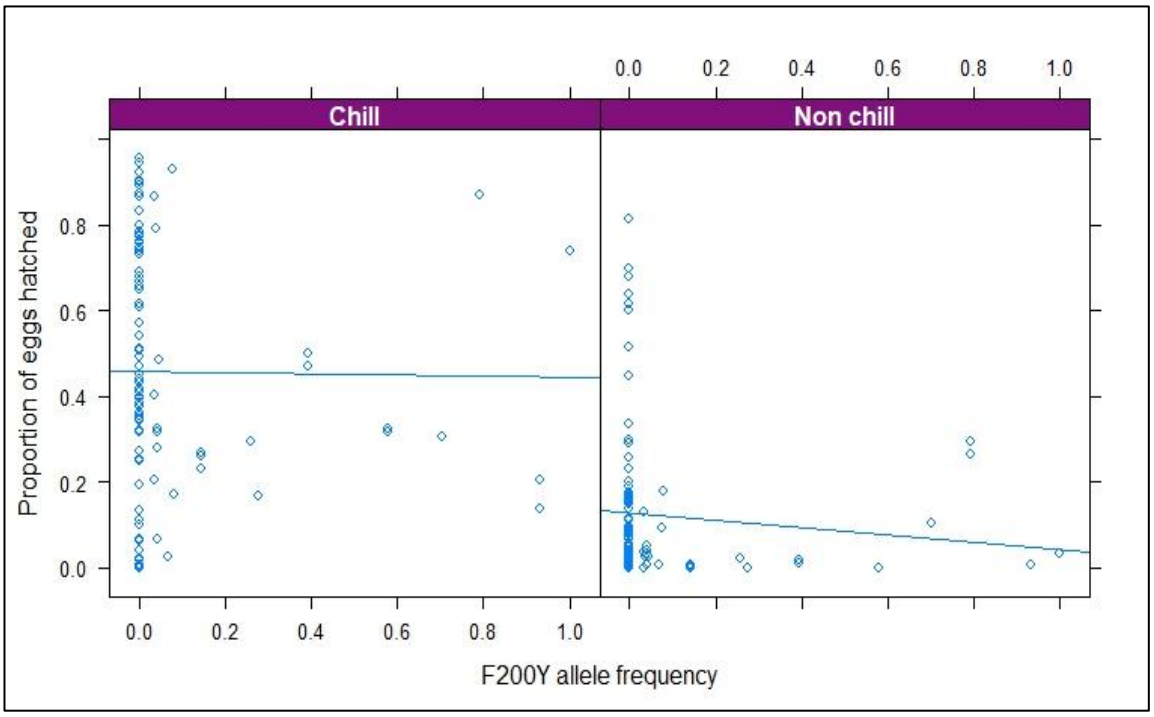


Figure 7.5. Comparison of F200Y resistant allele frequency and chill requirement of egg hatching. Comparison of the proportion of eggs hatched with and without a chill stimulus and the F200Y allele frequency of the isolate with regression lines.

7.4.2 Investigation of drivers

The impact of potential risk factors was assessed for hatching both with and without a chill in the current analysis. Previous studies have employed a 'propchill' method where the number of eggs hatched in the absence of a chill stimulus was assessed as a proportion of the number of eggs hatched under 'normal hatching conditions' i.e. following a chill (van Dijk and Morgan, 2010). Sample storage time at each stage of processing (i.e. storage time in faeces and egg development time post-extraction) was forced into each risk factor model to account for unavoidable variation in sample preparation between isolates. Completed management data was available from 32 of the 73 farms included in the hatching experiments.

7.4.2.1 Environmental factors

Initial GLMM analysis was performed using a range of environmental factors (Table 7.1). Chill stimulus, farm longitude (max-min) (-7.0-0.6) and latitude (50.8–58.2), maximum and minimum temperatures for spring and autumn (mean ±SEM (range)) (spring maximum; 14±0.07 (10.7–17.4), minimum; 6±0.05 (3.8–8.7), autumn maximum; 14±0.07 (10.3–17.5), minimum; 7±0.05 (5.0–10.1)), farm elevation 136±3.71 (4–383), precipitation 102±1.50 (50.3-165.9), average hours of sunshine 4±0.02 (2.9-4.6) and egg development time (incubation between egg extraction and experimental set up) 46±0.79 (15-98), were retained in the model as fixed effects with chill interaction terms for each, farm ID was also included as a random effect (Table 7.1).

Table 7.1. Model 1. Results of GLMM analysis with binomial outcome of hatching success in N. battus eggs (fitted with a logit link)). Covariates included were whether or not a chill stimulus was applied and a number of climatic variables; minimum/maximum temperature in spring/autumn, precipitation, number of hours of sun, farm elevation, farm position and experimental factors. Results are presented as odds ratios with 95% confidence intervals and p-values (Wald tests). Analysis included information from 80 farms.

Model covariates	Odds ratios	95% confidence interval		P-value
		Lower	Upper	
Non-chill	0.06	0.06	0.07	<0.0001
Longitude	0.57	0.39	0.84	0.002
Latitude	1.45	0.85	2.48	0.114
Spring maximum temperature	2.58	1.17	5.75	0.013
Spring minimum temperature	0.14	0.01	1.73	0.076
Autumn minimum temperature	2.45	0.36	16.62	0.219
Egg development time	1.00	1.00	1.01	0.426
Elevation	1.00	1.00	1.01	0.185
Precipitation	0.98	0.97	1.00	0.090
Average number of sun hours	2.19	0.18	26.46	0.521
Storage time	0.93	0.92	0.95	<0.0001
Non-chill interaction terms				
Latitude	0.84	0.74	0.95	0.005
Spring maximum temperature	0.70	0.60	0.81	<0.0001
Spring minimum temperature	4.22	2.424	7.41	<0.0001
Autumn minimum temperature	0.34	0.22	0.53	<0.0001
Egg development time	0.97	0.97	0.98	<0.0001
Elevation	1.00	1.00	1.00	<0.0001
Precipitation	1.01	1.01	1.01	<0.0001
Average number of sun hours	0.23	0.13	0.40	<0.0001
Storage time	1.02	1.01	1.03	0.019
Random effect				
Covariate	Residual variance			
Farm ID	1.371			

The climate model suggested that several of the geographic and climatic variables analysed had a statistically significant impact on non-chill hatching at the 5% level (Table 7.1). The 'non-chill interaction terms' describe how the impact of the covariate on non-chill hatching differs from that of chilled hatching. For example; farm latitude was positively associated with overall hatching (OR 1.45, 95% CI 0.85, 2.48, p=0.114) i.e. higher hatching in populations collected from farms in the north as these would have a higher latitude, the impact on non-chill hatching was significantly different from the overall effect; indicated by the significant interaction term (OR 0.84, 95% CI 0.74, 0.95, p= 0.005), therefore, non-chill was less strongly associated with latitude than overall hatching.

Spring temperature was found to have the greatest impact on hatching with and without prior chilling. Greater non-chill hatching was associated with spring temperature extremes i.e. high maximum and low minimum temperatures, suggesting that non-chill hatching may be associated with greater minimum-maximum intervals in spring temperatures. Minimum autumn temperature reduced the odds of non-chill hatching (OR 0.34, 95% CI 0.22, 0.53, p<0.0001) however, no significant interaction was identified between non-chill hatch and maximum autumn temperature at the 5% level. Hours of sunshine, a proxy for UV level was not significantly associated with overall hatching however, reduced the odds of egg hatching without chilling (OR 0.23, 95% CI 0.13, 0.40, p<0.0001) suggesting that non-chill hatching may be more common in regions with lower UV levels. The experimental set-up factors; sample storage and egg development time, were included in the model to account for variation in sample handling but neither was found to have a large impact on the number of eggs hatched with or without hatching (storage OR 0.93, 95% CI 0.92, 0.95, p<0.0001; OR 1.02, 95% CI 1.01, 1.03, p=0.003; egg development OR 1.00, 95% CI 1.00, 1.01, p=0.46; OR 0.97, 95% CI 0.97, 0.98, p<0.0001 with and without chilling respectively).

7.4.2.2 Management factors

Further GLMM analysis of the hatching data was conducted using management information and farm demographics collected by questionnaire (Table 7.2). The final model retained the following factors as fixed effects; chill stimulus, grazing strategy of lambs (set stocking (n=18), rotational grazing (n=10) or leader/follower (n=2)), away grazing (grazing of a proportion of lambs separate from the main farm each year, yes (n=20) or no (n=11)), resting and reseeding of high risk fields (i.e. fields which are grazed by 1-3 month old lambs each spring) (resting fields; yes = 25, no = 5, reseeding; yes = 4, no = 20), peak lambing week (mean ±SEM (range)) 14±0.22 (-2-21), faecal egg count of samples from which eggs were isolated (*N.* battus; 160±9.13 (1-605), and strongyle nematode species; 169±13.61 (0-1435)) and storage time of eggs in faeces (i.e. time from faecal collection to egg extraction); 11±0.35 (1-33) (Table 7.2). Farm ID was also included as a random effect as previously described.

Table 7.2. Model 2. Results of GLMM analysis with binomial outcome of hatching success in N. battus eggs (fitted with a logit link). Covariates included were whether or not a chill stimulus was applied and a number of farm management practices; away grazing, grazing strategy of lambs, resting and reseeding highly contaminated fields, peak lambing week, faecal egg count of the faecal samples from which eggs were extracted and experimental factors. Results are presented as odds ratios with 95% confidence intervals and p-values (Wald tests). Analysis included information from 32 farms.

Model covariates		Odds ratios	95% confidence interval		P-value
			Lower	Upper	
Non-chill		0.02	0.01	0.04	<0.0001
'Away grazing'		0.92	0.07	12.53	0.95
Grazing strategy	Rotational	0.96	0.02	44.98	0.98
	Set stocking	19.89	0.67	602.86	0.07
Sample storage		0.90	0.88	0.92	<0.0001
Egg development time		0.99	0.98	1.00	0.14
Rest fields		0.43	0.02	9.24	0.57
Reseed fields		3.91	0.09	180.89	0.46
Peak lambing week		1.47	1.16	1.90	0.002
***N. battus* faecal egg count**		1.00	1.00	1.00	0.44
Strongyle faecal egg count		1.01	1.01	1.01	<0.0001
Non-chill interaction terms					
'Away grazing'		1.97	1.03	2.80	<0.0001
Grazing strategy	Rotational	2.73	1.94	6.96	0.02
	Set stocking	4.51	2.47	11.86	0.001
Sample storage		1.10	0.13	1.13	<0.0001
Egg development time		1.00	0.02	1.02	0.56
Rest fields		10.89	2.77	15.97	<0.0001
Reseed fields		2.30	1.40	4.05	0.004
Peak lambing week		1.19	0.24	1.27	<0.0001
***N. battus* faecal egg count**		1.01	0.00	1.01	<0.0001
Random Effect					
Covariate		Residual variance			
Farm ID		6.009			

GLMM analysis omits data points which do not contain values for all factors included in the model. Modelling of hatch data with climatic factors was based on information from 80 farms. A total of 40 completed management questionnaires were collected from the study farms. The addition of management factors reduced the number of data points included in the model to 32 farm populations.

Hatching of eggs without prior chilling was found to be 10 times more likely on farms which rested fields posing a high *N. battus* risk (i.e. heavily infected fields repeatedly grazed by 1-3 month old lambs each spring) compared to those with permanent pasture (OR 10.89, 95% CI 2.77, 15.97, p<0.0001). The odds of non-chill hatching were also increased on farms which reseeded 'high risk' fields (OR 2.30, 95% CI 1.40, 4.05, p=0.004).

Non-chill hatching was found to be associated with the grazing strategy of lambs; *N. battus* populations collected from farms which set stocked lambs were 4 times more likely to hatch without chilling compared to farms which employed cellular or leader/follower systems (OR 4.51, 95% CI 2.47, 11.86, p=0.001). Away grazing was also found to double the odds of eggs hatching without prior chilling (OR 1.97, 95% CI 1.03, 2.80, p<0.0001).

Sample storage and egg development time during experimental set up were included as previously described. Time in faeces was associated with a reduction in overall hatch (OR 0.90, 95% CI 0.88, 0.92, p<0.0001) and an increase in non-chill hatching (OR 1.10, 95% CI 0.13, 1.13, p<0.0001). Egg development time, post-extraction, was not found to have a significant impact on the number of eggs hatched with or without chilling (OR 0.99, 95% CI 0.98, 1.00, p = 0.14 and OR 1.00, 95% CI 0.02, 1.02, p = 0.56 with and without chilling).

The faecal egg count of the original samples from which the study populations were isolated were included in the model as a proxy for infection level and co-infection status. Strongyle species egg count was found to have a small impact on the proportion of eggs hatched

following a chill stimulus (OR 1.01, 95% CI 1.01, 1.01, p<0.0001). However, the association between non-chill hatching and strongyle faecal egg count was not significant at the 5% level, perhaps suggesting a temporal aspect to the requirement for chilling prior to hatching in *N. battus* eggs. *N. battus* egg count was found to have a significant interaction with non-chill hatching i.e. it explained a proportion of the variation observed however, had little impact on the odds of eggs hatching with or without chilling (OR 1.01, 95% CI 0.00, 1.01, p<0.0001).

Samples were collected from lambs born between the beginning of January and mid-May, average early April. Peak lambing week, collected by farmer questionnaire and coded by week of the year, was identified as having an influence on the number of eggs hatching with and without a chill stimulus (OR 1.47, 95% CI 1.16, 1.90, p = 0.002 and OR 1.19, 95% CI 0.24, 1.27, p<0.0001 with and without chilling respectively); suggesting that a higher proportion of eggs hatched from samples collected from lambs which were born later and would therefore be younger at the point of infection. Figure 7.6 illustrates the pattern of egg hatching observed with varying lamb age at sampling and shows a higher proportion of eggs hatched without chilling in populations isolated from younger lambs, reducing as lamb age increased whilst chilled populations maintained a high level of hatching. The plot highlights a change in the hatch profile around 16 weeks of age where the difference in the proportion of eggs hatched with and without chilling becomes much greater.

Peak lambing week was confounded by both lamb age and sample collection date, all were included during model development however, the model including peak lambing week was found to be the best fit for the observed data.

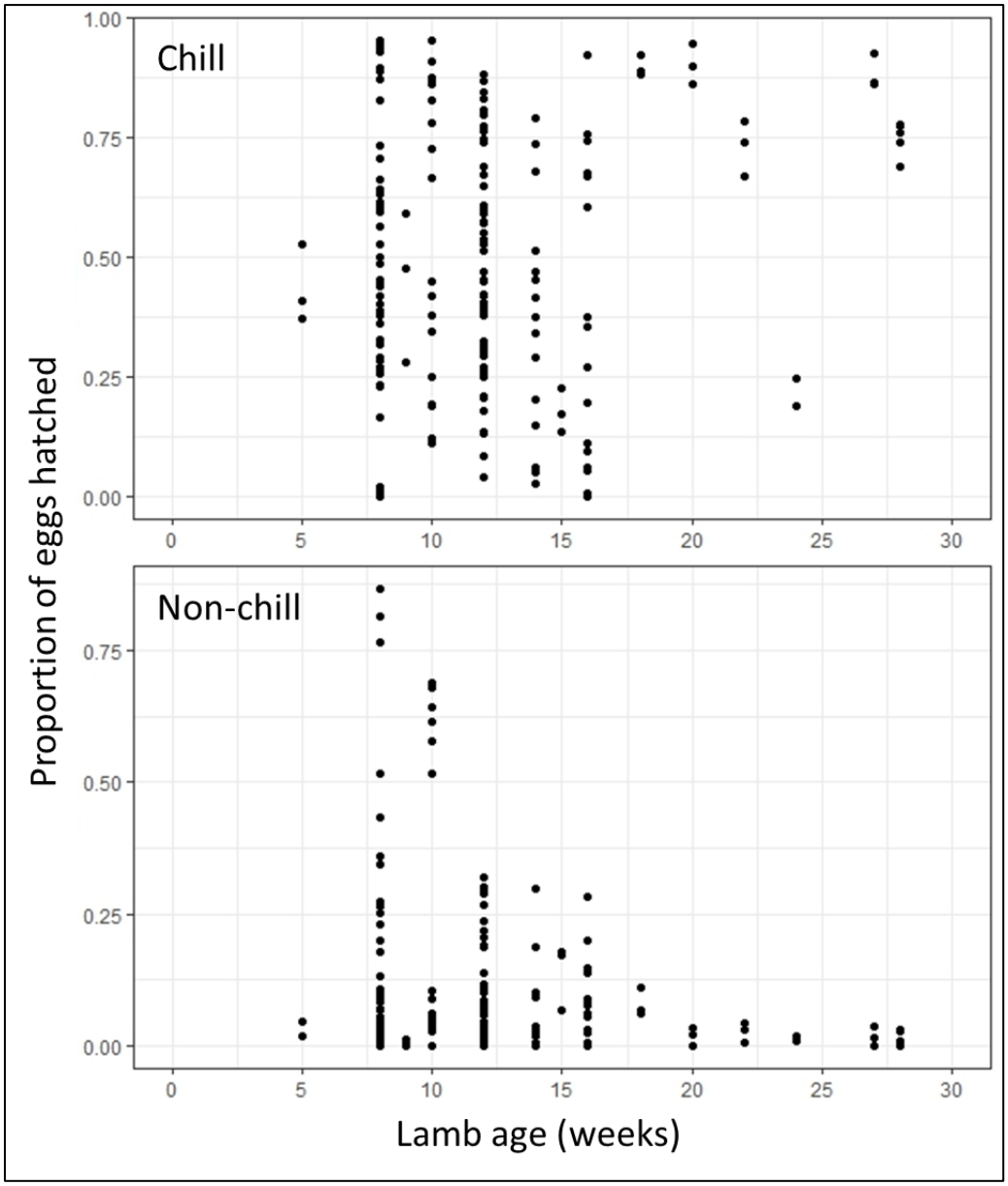


Figure 7.6. Comparison of lamb age at sampling and the requirement for chilling in N. battus egg hatching.

Proportion of eggs hatched in each population, with (top) and without (bottom) a period of chilling vs. lamb age (weeks).

7.4.2.3 Host factors

A third model was developed to explore the impact of farm type and lambing week (Table 7.3). Chill stimulus, farm type (lowland (n=25), upland (n=13) and hill (n=9)), peak lambing week (mean ±SEM (range)) 14±0.22 (-2-21) and storage time of eggs in faeces (11±0.35 (1-33)) were retained as fixed effects with farm ID as a random effect (Table 7.3). When split by farm type, the hatching profile of eggs varied between lowland, upland and hill populations (Figure 7.7). Hatching of *N. battus* eggs was higher in lowland farm populations compared to those collected from hill farms following a chill stimulus (OR 3.06, 95% CI 1.07, 8.81, p= 0.043) however, the opposite was observed in non-chill populations (OR 0.40, 95% CI 0.30, 0.53, p<0.0001). The impact of lambing week on hatching differed between farm types (OR 0.79, 95% CI 0.62, 0.99, p=0.217, comparing lowland and hill populations) and the complex lambing week-farm type interaction differed between chill and non-chill models (OR 1.10, 95% CI 0.98, 1.23, p=0.141) but these interactions were not statistically significant given the current data set. Due to the random sampling method used, no samples were available from animals born prior to weeks 12 and 9 from upland and hill flocks respectively. Predictions from model 3 (Figure 7.7) illustrated the influence of peak lambing week on the proportion of eggs hatching with and without a chill stimulus differs by farm type. Chilling appeared to increase the magnitude of hatching on hill farms but the timing of lambing had a similar influence regardless of chilling in these populations; a higher proportion of eggs hatched with later lambing date. Predictions for upland and lowland farms showed a lower proportion of eggs hatched with later lambing under chill conditions; this effect appeared strongest in upland farms. Non-chill predictions highlighted a different hatching profile for each farm type. Lowland farms were found to have a similar non-chill hatching profile as hill farms although, the influence of lambing date was stronger on hill farms. Lambing date was predicted to have a lower impact on non-chill hatching of eggs on upland farms compared to lowland and hill systems and the proportion of eggs hatching under non-chill conditions was

predicted to be lower in samples collected from late born lambs on upland farms. Sample storage time in faeces was again, negatively associated with overall hatching but increased the odds of non-chill hatching (OR 0.92, 95% CI 0.91, 0.94, p<0.0001 and OR 1.04, 95% CI 1.02, 1.05, p<0.0001 for hatching with and without a chill stimulus respectively). The impact of egg development time was marginal but was found to be associated with a small reduction in the proportion of eggs hatched without chilling (OR 1.00, 95% CI 0.99, 1.01, p=0.818 and OR 0.97, 95% CI 0.96, 0.97, p<0.0001 for hatching with and without chilling respectively). The impact of storage time in faeces can be seen in the chill predictions (run using mean storage time ±1 day) and appears to have a similar impact on hatching proportions from each farm type however, storage time in faeces was found to have minimal impact on non-chill hatching and predicted points cluster together.

Table 7.3. Model 3. Results of GLMM analysis with binomial outcome of hatching success in N. battus eggs (fitted with a logit link). Covariates included were whether or not a chill stimulus was applied, peak lambing week, farm type and experimental factors. Results are presented as odds ratios with 95% confidence intervals and p-values (Wald tests). Analysis included information from 52 farms.

Model covariates		Odds ratios	95% confidence interval		P-value
			Lower	Upper	
Non-chill		0.15	0.12	0.19	<0.0001
Peak lambing week		1.18	0.96	1.49	0.163
Farm type – upland		1.77	0.55	5.75	0.911
Farm type – Lowland		3.06	1.07	8.81	0.043
Sample storage time (in faeces)		0.92	0.91	0.94	<0.0001
Egg development time		1.00	0.99	1.01	0.818
Interaction terms					
Non-chill / Peak lambing week		1.08	0.98	1.21	0.098
Non-chill / Farm type – upland		0.59	0.45	0.80	0.446
Non-chill / Farm type – lowland		0.40	0.30	0.53	<0.0001
Non-chill / Sample storage time		1.04	1.02	1.05	<0.0001
Non-chill / Egg development time		0.97	0.96	0.97	<0.0001
Peak lambing week / farm type	Upland	0.68	0.43	1.06	0.108
	Lowland	0.79	0.62	0.99	0.217
Non-chill / Peak lambing week / farm type	Upland	1.24	1.08	1.42	0.001
	Lowland	1.10	0.98	1.23	0.141
Random Effect					
Covariate		Residual variance			
Farm ID		1.536			

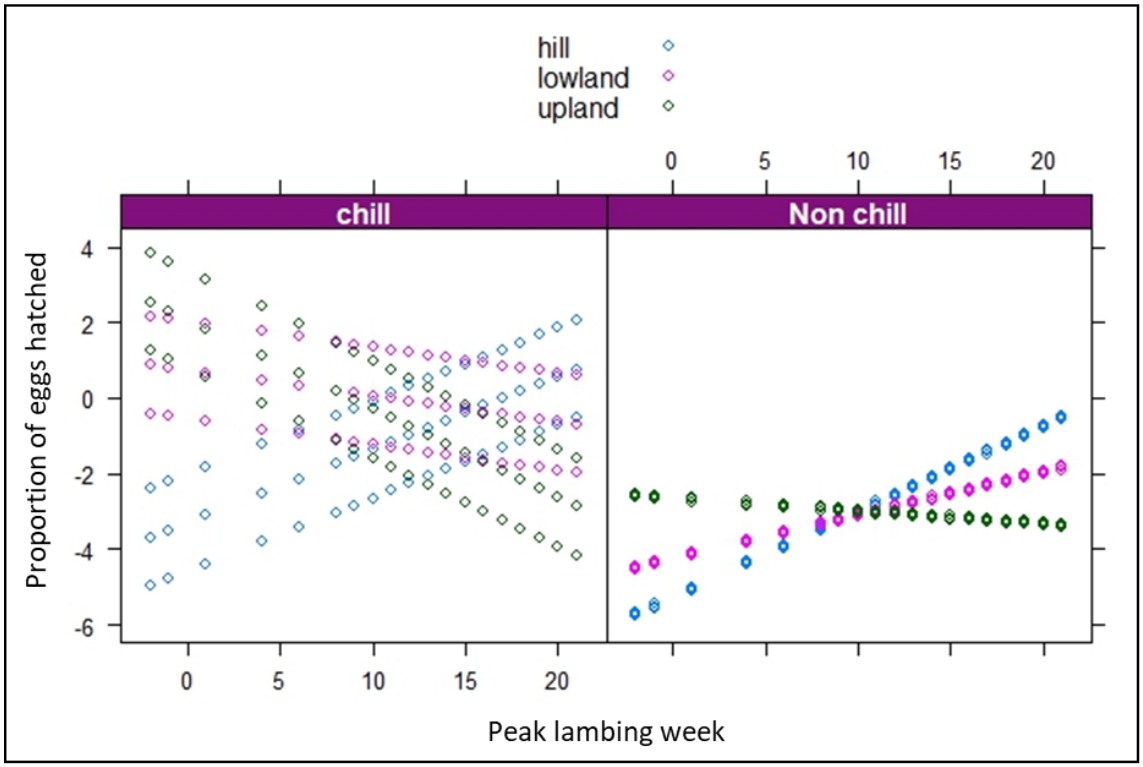


Figure 7.7. Predictions from model 3.

Predications illustrate the differential impact of peak lambing week (range -2-21) on the estimated proportion of eggs hatching with and without a chill stimulus from lowland (pink), upland (green) and hill farms (blue).

Triplicate points in chill predictions represent variation in egg storage time in faeces (mean±1 day).

7.4.2.4 Model comparison

Models 1 and 2 were compared using a likelihood ratio test which determined that both models explained a similar amount to the variation observed between populations in the proportion of eggs hatched with and without prior chilling (χ^2= 0, d.f.= 1, p= 1). Comparison of models 2 and 3 selected model 2 as a better explanation of the observed data (χ^2= 717.9, d.f. = 6, $p<2.2x10^{-16}$). Despite models 1 and 2 fitting the data better, model 3 was also reported to highlight possible variation in the impact of certain drivers between farm types. All model outcomes should be verified using field tests i.e. experimental infections where a single factor is varied to study the effect of key management or climatic factors in isolation.

The findings of model 3 also highlighted variation between farm types so duplicate tests conducted in a number of different farm settings may be required.

7.5 Discussion

A sizeable proportion of *N. battus* eggs successfully hatched without a chill stimulus in the current study. Traditionally, *N. battus* eggs were believed to require a period of chilling before biologically significant levels of hatching occurred (Thomas and Stevens, 1960). Extended exposure of eggs to low temperatures was believed to be integral to the conversion of energy reserves within larvae from lipids to sugars which has been shown to increase both cold-hardiness (Ash and Atkinson, 1983) and larval longevity on pasture (van Dijk and Morgan, 2010). Hatching in the absence of a chill has been previously documented, albeit at low levels, both historically (Boag and Thomas, 1975; Gibson and Everett, 1981; Thomas and Stevens, 1960) and more recently (Sargison et al., 2012; van Dijk and Morgan, 2008; van Dijk and Morgan, 2010). Our analyses suggest that the control of hatching in *N. battus* is multifactorial; driven by a complex interaction of environmental, farm management and host-parasite variables.

The three models described identified a number of factors associated with the proportion of *N. battus* eggs hatched with and without a chill stimulus. In agreement with previous research findings, spring temperature range was found to have the greatest influence of the environmental factors. However, this effect was stronger with relation to the hatching of eggs after chilling. Analysis of management factors in model two found egg hatching in the absence of a chill stimulus was associated with resting and reseeding of fields typically grazed by 1-3 month old lambs in spring, likely due to interruption of the *N. battus* lifecycle. Model three identified differences in *N. battus* egg hatching profiles between farm types. The results suggested that the influence of management factors such as lambing date on the requirement for a chill stimulus in eggs may vary between lowland, upland and hill farms.

The number of hatched eggs increased with the addition of a chill stimulus in the majority of populations tested, indicating that a number of eggs still required a chill to hatch as previously reported. Non-chill hatching was greater than chilled hatching in seven populations tested, perhaps indicating that hatching without prior chilling may now be the predominant mechanism on a small proportion of farms. Analysis was therefore conducted on chilled and non-chilled results rather than propchill (the number of eggs hatched without chilling, given as a proportion of the number of eggs hatched following a chill from the same population) as in previous studies (Ash and Atkinson, 1983). Large variation in the proportion of eggs hatched after chilling was observed between isolates, perhaps be due to haplotype variation between populations. Although testing of genetic variation was out-with the remit of the present study, further analysis of the DNA sequence data generated from these populations during MiSeq analysis in chapter 4 may be possible in the future.

Variation in experimental set up was included in the model to account for variation between populations which would otherwise have been negated by analysing propchill therefore both storage time in faeces and egg development time following extraction were forced into the models. The impact of storage and egg development time on hatching with and without chilling was illustrated by the predictions from model 3 (Figure 7.7). Sample storage time, both within faeces and following egg extraction, was identified as having a statistically significant impact upon the proportion of eggs hatched after chilling; a lower proportion of eggs hatched with increasing storage time in faeces, in line with previous accounts of egg viability (Gibson and Everett, 1981). Variation in sample storage time was un-avoidable due to the labour-intensive process of sample collection and processing. The influence of sample processing and experimental conditions on hatching percentages, emphasises the need for detailed standardised egg processing protocols for such experiments.

Multiple models were built to assess the influence of farm management, environmental and host-related factors on hatching whilst reducing the impact of confounding variables where possible. For example, climatic factors such as temperature and precipitation were confounded with geographic region, time of year and co-infection with strongyle nematode species. By developing multiple models, it allowed for detailed analysis of all factors.

Several factors highlighted by the current analysis were in agreement with existing hypotheses and previous studies, including the importance of spring temperature range (van Dijk and Morgan, 2008) and the lesser impact of precipitation (van Dijk and Morgan, 2012) on hatching in this species. The results indicated a geographical trend; a significantly higher proportion of eggs hatched in Scottish isolates compared to those collected from England and Wales, both with and without a chill stimulus. In a previous study comparing hatching of four *N. battus* isolates, higher chilled hatching was again observed in Scottish compared to English populations however, the opposite was reported for non-chill hatching (van Dijk and Morgan, 2010). Higher hatching without chilling in the north may appear counter-intuitive, given the reliability of cold temperatures in the north and predictions of *N. battus* spring hatch being more consistent in Scotland compared to southern regions in the face of climatic warming (Gethings et al., 2015). However, hatching is likely to be influenced by a number of environmental and management factors. The current analysis also highlighted a number of novel drivers associated with *N. battus* egg hatching including the impact of the timing of lambing and infection level of the host (Figure 7.8).

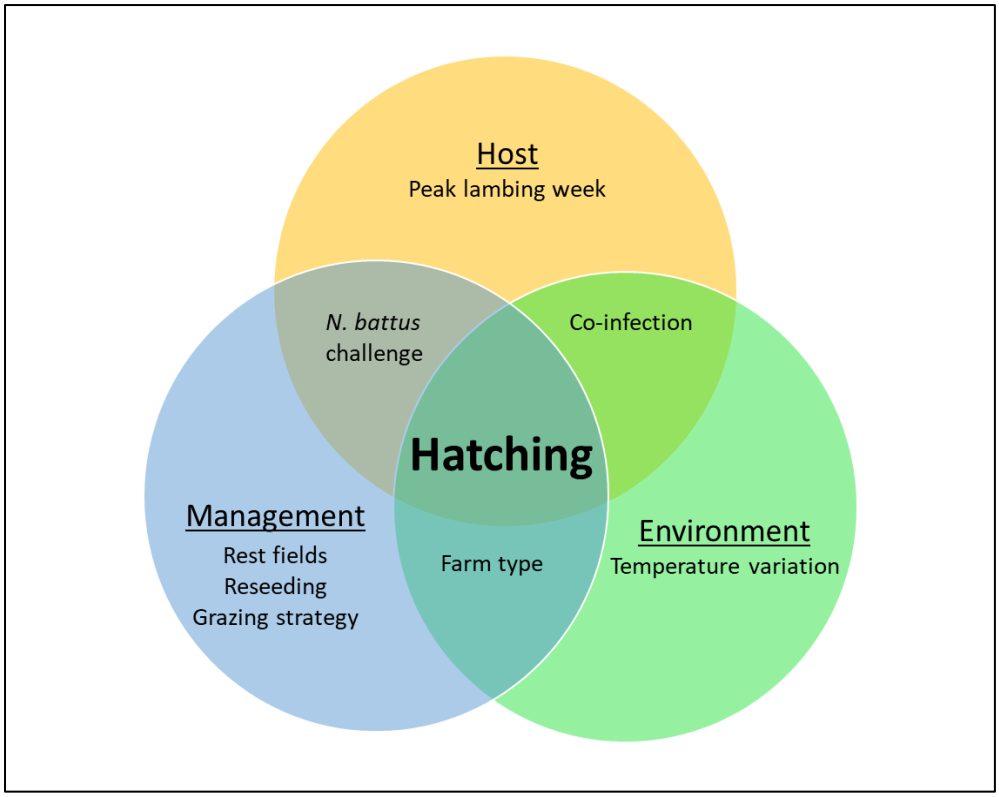


Figure 7.8. Factors highlighted by the current analysis as having a significant impact on the hatching of N. battus eggs in vitro

It was hypothesised that the emergence of benzimidazole resistance may be associated with the hatching of *N. battus* eggs out-with the traditional spring hatch, suggesting that populations which are active throughout the grazing season may be exposed to a greater number of anthelmintic treatments. No statistically significant correlation was identified between resistant allele frequency (F200Y) and the proportion of eggs hatched with or without a chill stimulus. Non-chill hatching is therefore unlikely to be a significant driver of the emergence of BZ-resistance in the species, as discussed in chapter 6. Variable hatching of eggs may reduce the selection pressure from treatment as not all larvae would be exposed at any one time. The lack of association between the proportion of eggs hatched after chilling and resistant allele frequency suggests that the mutation is unlikely to carry a significant fitness cost affecting egg development, in agreement with BZ-resistance in other trichostrongylid species (Elard et al., 1998).

7.5.1 Impact of environmental factors

Spring temperature range was found to influence hatching with and without chilling and additional associations were identified between non-chill hatching and precipitation, latitude and average number of hours of sunshine (model one).

Previous studies have highlighted the influence of spring temperature on the hatching rate of *N. battus* (van Dijk and Morgan, 2008; van Dijk and Morgan, 2010) and the findings of this analysis were in agreement, indicating that high spring temperature range (i.e. high maximum and low minimum temperatures) may be associated with increased overall hatching in this species. The impact on non-chill hatching was lower than chilled hatching however, it was still statistically significant. It has been hypothesised that high temperatures or highly variable temperatures may stimulate larvae to become hyperactive which could accelerate hatching (van Dijk and Morgan, 2008). Links have also been suggested between variable spring temperatures and bet-hedging; hatching of a proportion of eggs at an atypical time or conditions in an attempt to maximise or safeguard transmission despite environmental variability (van Dijk and Morgan, 2010). Increased hatching without chilling may be driven by variable spring conditions. In years where spring temperature is variable or increases rapidly so that a proportion of eggs are prevented from hatching in spring (van Dijk and Morgan, 2008), non-chill hatching could be advantageous as lambs may be less likely to have acquired strong immune protection given the reduced spring peak challenge therefore potentially remaining susceptible to infection later in the grazing season. Results of the current analysis suggest that spring temperature is important in *N. battus* hatching overall but may not be as important a driver of non-chill hatching as previously suggested. Non-chill hatching was also associated with reduced hours of sunshine (a proxy for UV exposure). As *N. battus* larvae are highly sensitive to UV (Van Dijk et al., 2009), reduced sunlight would therefore increase larval survival time on herbage and maximise transmission opportunities.

Prior research on the impact of moisture on hatching of *N. battus* eggs concluded that, whilst water was required, this was unlikely to be a key driving force (van Dijk and Morgan, 2012). Studies based on the moisture content in soil identified that *N. battus* hatching occurred at a slower rate and to a lower extent in high moisture content, nearing field capacity, and at particularly low moisture content, nearing wilting point (Parkin, 1975). As UK conditions do not typically near either arid, wilting point or field capacity out-with extreme weather events, it could be assumed that precipitation levels are generally similar to the mid-point moisture level used in Parkin's study which resulted in increased hatching. Results of the current analysis were in agreement, suggesting that the influence of precipitation on the ability of eggs to hatch without chilling was minimal. Similarly, elevation and average number of hours of sunshine were shown to be non-significant factors.

7.5.2 Impact of management factors

Management factors were not found to influence chilled hatching however, several factors were significantly linked with non-chill hatching, suggesting that management strategies may be important drivers of egg hatching without prior chilling.

Resting and reseeding of 'high risk' fields (land repeatedly grazed by lambs under 3 months old each spring) and away grazing (grazing lambs separate from the main farm for a proportion of the year) were each found to be positively associated with non-chill hatching. The association may represent a bet hedging approach to hatching; thought to be driven by uncertainty and variation (Meyers and Bull, 2002) as reseeding, resting fields and 'away grazing' all introduce uncertainty in host availability. Another possible explanation could be low parasite refugia; reseeding and resting fields are practices often employed to reduce parasite contamination on pasture. Re-expansion of the *N. battus* population following resting or reseeding could occur from small numbers of eggs/larvae which, if the requirement for a chill stimulus is genetically driven, could be predisposed to hatch under

certain conditions i.e. without a chill. Alternatively, reduction in population size could influence the hatching requirements of eggs via a density dependent mechanism. Density-dependent development has been documented in seed germination studies, suggesting that bet-hedging occurs in desert annual plant communities, dependent upon the number of plants and water availability to maximise survival (Gremer and Venable, 2014). It may be possible that *N. battus* is capable of employing a similar strategy to maximise transmission opportunities.

N. battus and strongyle faecal egg counts were included as proxies of host infection level and co-infection. Chilled hatching was associated with increased strongyle egg count whilst non-chill hatching was linked with *N. battus* egg count, albeit with minimal predicted impact on the odds of eggs hatching with or without chilling. These results may suggest a positive effect of time of year, lamb age or density dependent factors (as discussed above). Given the typical progression of nematode species throughout the grazing season (Melville et al., 2016), the results suggest that non-chill hatching is more likely from eggs passed out early in the season from young lambs. Eggs from older lambs co-infected with strongyle species in summer would be more likely to require a chill stimulus to hatch. Indeed, the hatching behaviour of the species *N. filicollis* has been proposed to be influenced by density dependence and co-infection patterns (Keymer, 1982; van Dijk and Morgan, 2009). Interspecies interactions have also been hypothesised many times (Ferrari et al., 2009; Knowles et al., 2013). Alteration of physiological conditions such as pH of the gastrointestinal tract have been documented (Mapes and Coop, 1970) but the nature and extent of interspecies communication and interaction are still poorly understood. It may be possible that co-infection with strongyle species as the grazing season progresses may alter the physiological conditions in the gut, influencing the hatching requirements of eggs e.g. to require a chill stimulus.

Grazing strategy was also highlighted by the analysis; suggesting that non-chill hatching was more likely in set stocked lambs than those which were grazed in a leader/follower system. Despite contradicting the proposed bet-hedging hypothesis, set stocking may select for non-chill hatching by providing hosts for autumn hatched larvae albeit with an anticipated lower 'success' rate as the lambs will be partially immune. Although the estimated impact of set stocking on non-chill hatching was high, this describes the likelihood of non-chill hatching in this system compared to the referent value which, in this case, was a leader/follower grazing system which was used by few farms in the current study, thus the result may be skewed. Also, respondents were not asked whether set stocked lambs were grazed on the same permanent pasture each year, this would likely have a significant impact on *N. battus* hatching behaviour so the true influence of set stocking cannot be determined from the current results.

7.5.3 Impact of host factors

Current analysis identified the timing of lambing as having an impact on *N. battus* egg hatching. Peak lambing date was obtained from farm management questionnaires and was coded as week of the year. Both lambing date and lamb age at sampling were considered during model development however the former was retained as it provided a better explanation of the variability observed. Lambing week was found to have a significant impact on hatching both with and without prior chilling however, it differed between farm types. Lambing date had a positive association with the proportion of eggs hatching on hill farms regardless of chilling however, on lowland farms, the impact of lambing date on egg hatching differed dependent on the presence of a chill stimulus. The variation in hatching profiles observed between the different farm types do not appear to be directly correlated with elevation therefore, other factors which differ between lowland, upland and hill farms must be responsible for the variation predicted such as breed differences or additional management practices.

Gethings *et al.* (2015) predicted that the timing of the peak *N. battus* egg hatch would shift earlier in the year due to climate warming, potentially before naïve lambs would be grazing thus, providing a phenological mismatch between host and parasite. Farms which lamb sheep later will be more likely to miss the peak *N. battus* egg hatch in spring and therefore avoid the rapid development of strong immunity. The current analysis highlighted late lambing as a potential driver of non-chill hatching which is in agreement with Gething's predictions, increasing the likelihood of hatch and grazing mismatch therefore increasing the potential success of *N. battus* infection in autumn from non-chill hatched eggs. The association with non-chill hatching and late lambing may also indicate that immune factors could influence the resultant hatching behaviour of eggs, as observed in *Strongyloides* species where the development of host immunity has been shown to alter the reproductive strategy of subsequent adult morphs (Gemmill et al., 1997; Harvey et al., 2000).

Development of immunity in lambs results in the exclusion of adult worms from the small intestine (Taylor and Thomas, 1986), therefore the reduction in parasite numbers may influence the fate of eggs via a density-dependent pathway, as was hypothesised from the link with *N. battus* faecal egg count. The strong association observed between strongyle nematode egg count and chill hatching adds further support to the theory that the requirement for a chill stimulus may change throughout the course of the infection, as strongyle species are typically found later in the grazing season. Lambs are thought to develop a degree of age-related immunity (Winter et al., 1997a) which may explain the suppression in non-chill hatching observed from around 16 weeks of age. Each sample only represents a snapshot of the population so it is impossible to determine, from the current data, whether the effect observed is due to lamb age or another factor. Longitudinal sampling of a single population throughout the grazing season with repeated hatching tests would be required to study the impact of lamb age on hatching and could also provide

additional insight into the possible role of immune factors in the control of hatching and the requirement for a chill stimulus.

Differences were observed in the hatching profile of eggs collected from lowland and hill farms; a greater magnitude of hatch was observed with lowland compared to hill populations following chilling, in agreement with previous findings (Thomas, 1990). The impact of lambing week on hatching requirements also appeared to vary both between farm types and between chill and non-chill models. Non-chill hatching was higher in populations collected from hill farms compared to lowland, this followed a previous findings (van Dijk and Morgan, 2010). Differences at the farm type level could be due to a wide range of factors. Parasites on hill farms may favour a bet-hedging approach to hatching to increase the chance of successful transmission due to variability in host availability, and potentially in climatic conditions. One difference between the study farms was the breed of sheep kept; lowland farms typically stocked Texel, Suffolk and cross bred mules, hill farms stocked largely traditional hill breeds such as Swaledale and Scottish Blackface. Sheep breeds are known to vary in their resilience to nematode infection; previously illustrated both with natural infection of Texel and Suffolk lambs and experimental studies with *Haemonchus contortus* comparing wool and hair breeds (Good et al., 2006; Terefe et al., 2007). Little is known about the influence of breed on immune development to *N. battus* infection however, it is conceivable that some differences may exist. As the environmental challenges of farming vary between lowland, upland and hill farms, management strategies are altered to fit the requirements of each system. A previous study reported a 3 month delay in the timing of peak hatch observed in a *N. battus* isolate collected from a hill flock compared to a lowland population when all eggs were placed under the same environmental conditions (Thomas, 1990). It may be possible that eggs which failed to hatch in the present study may have hatched if incubated for longer however, this was out-with the scope of the present study

and previous work indicated that hatching typically plateaued within 4 weeks given the appropriate hatching conditions (van Dijk and Morgan, 2010).

The mechanism of adaptive variation in *N. battus* hatching remains unclear. The requirement for a chill stimulus may be under genetic control and as such, can be selected for over time. Alternatively, hatching may follow a bet-hedging approach where genotypically similar eggs are stimulated to hatch with or without chilling by factors within the host (e.g. immune factors or parasite signals) or the environment. Larvae from eggs hatched with and without chilling could be used in experimental sheep infections to assess the genetic control of the requirement for chilling in *N. battus* eggs.

Results obtained from this analysis suggested that farm management, density dependence, immunological factors and environmental factors may all either provide cues or influence the hatching behaviour of *N. battus* through selective advantage of offspring. Stimulation of eggs to hatch with or without chilling by external factors suggests that hatching is a plastic attribute which may vary between neighbouring populations, year to year or throughout a single grazing season to allow adaptation to the present environment. The impact of host age and immune status on the requirement for chilling in *N. battus* eggs is an important research question. Detailed knowledge of the expected timing of egg hatching and the factors which influence it are vital to inform prediction models and effective control strategies for *N. battus.* The current study examined a snap-shot from each population to explore the requirement for a chill stimulus at the individual and population levels. Close monitoring of a smaller number of populations throughout the grazing season using repeated hatching experiments would provide valuable information on hatching dynamics over time.

7.6 Conclusion

Hatching behaviour in *N. battus* appears to be a plastic attribute, influenced by a combination of climatic, farm management and possibly immunological factors, varying between populations and possibly within and between grazing seasons. Resting and reseeding of 'high risk' fields were identified as the strongest single influence on non-chill hatching, suggesting that farm management factors may have a bigger role to play than previously thought. Investigation of the possible genetic control of the requirement for chilling in *N. battus* eggs and exploration of the impact of host age and immune status on egg hatching throughout the grazing season would be valuable next steps. Further investigation would provide insight into the control of hatching and the potential impact of drivers identified in the current study which may allow for the development of tailored on-farm control strategies.

8 General discussion

Since the initial characterisation of *Nematodirus battus* in the 1950s (Crofton and Thomas, 1951) this parasite species has become more difficult to control due to less predictable epidemiology and the recent emergence of anthelmintic resistance. The aims of this project were to explore the novel resistance-associated changes observed in some *N. battus* populations; quantify BZ-resistance and hatching patterns throughout the UK and to explore current control measures to assess whether farm management practices may be driving the changes in epidemiology or emergence of resistance observed. *N. battus* remains an important threat to lamb health in the UK. Interest from the agricultural community was evident in the large number of samples volunteered for analysis and queries at agricultural shows and animal health talks throughout the span of the project. The enthusiasm from the livestock sector highlighted the importance of the work and the need for updated information on this topic. It is important to understand the novel aspects of parasite epidemiology to design effective control strategies, minimise production loss and protect animal welfare.

To our knowledge, this project was the largest survey of UK *N. battus* populations conducted to date, the large number of populations included adds power to the findings, reducing sampling bias. A range of applied parasitology techniques were used to collect, isolate and culture *N. battus* populations and a range of state-of-the-art molecular genotyping methods were designed and evaluated for the detection and quantification of SNPs associated with BZ-resistance. The laboratory-based Illumina MiSeq and pyrosequencing methods were both found to be suitable for use as diagnostics or research tools however, development of a rapid diagnostic test using loop-mediated isothermal amplification proved difficult within the time constraints of the current project. F200Y was identified as the main SNP associated with BZ-resistance in *N. battus.* At the farm level, in terms of presence-absence, the mutation was

found to be highly prevalent; identified in ¼ of the populations tested. However, currently, the allele frequency remains low on most farms. A focal region, in which several local populations possessed a higher than average F200Y allele frequency, was identified in North West England, surrounding the farm where the initial BZ-resistant *N. battus* isolate was identified. The origin(s) and likely spread of resistant alleles throughout the country cannot be reliably inferred from prevalence data however, further analysis of the sequence data produced from MiSeq analysis during the current project could provide an opportunity to explore the origin and spread of anthelmintic resistance. Exploration of BZ-resistance development in other nematode species has proven difficult due to the high prevalence of resistance in multiple countries (Chaudhry et al., 2015b; Redman et al., 2015; Silvestre et al., 2009; Skuce et al., 2010) however, as BZ-resistance appears to be at an early stage in *N. battus* currently, this may be the ideal model system for study. *N. battus* remains a significant threat to lamb health in the UK and with limited anthelmintic options available for safe treatment of young stock, it is pertinent to monitor benzimidazole resistance in this species in an attempt to slow the spread of resistance and safeguard benzimidazole efficacy. Due to the significant differences in life history traits and epidemiology between *N. battus* and other trichostrongylid species, knowledge gained from the study of *N. battus* is not directly transferrable to other species. However, the changes in epidemiology observed, towards more year-round activity, may result in this species becoming more similar to other trichostrongylids, including other *Nematodirus* species, at which point information may be more transferrable. With continued variation in *N. battus* epidemiology then BZ-resistance in this species may advance in line with *N. spathiger* in 5-10 years time.

Hatching experiments were conducted to quantify the proportion of eggs which required a chill stimulus for successful hatching in different populations, a proxy for epidemiological variation in hatching patterns. The proportion of eggs able to hatch without prior chilling varied greatly between populations and was found to have a geographic trend; a greater

proportion of eggs collected from farms in Scotland were able to hatch without a chill stimulus compared to those from the South. The reason for the greater variability observed in the North remains unclear. Given that temperature conditions are typically favourable for *N. battus* in Scotland and disease outbreaks seem to be concentrated more in spring, additional research is required to understand the factors controlling the timing of *N. battus* egg hatching. Researchers have reported similar hatching without chilling in a small proportion of *N. filicollis* populations (Oliver et al., 2014; Oliver et al., 2016b). The variability in hatching requirements observed in some *Nematodirus* populations may be responsible for the apparent expansion of these species into novel environments including reports of *N. filicollis* in the sub-humid tropics of Mexico (Rodriguez-Vivas et al., 2017) and *N. battus* in Sicily (Torina et al., 2004).

Providing information on the prevalence of anthelmintic resistance or the ability of eggs to hatch under different conditions is interesting and allows for progression to be measured over time but this information is of limited use without understanding the drivers involved. This project explored current farm management practices and the association of different factors with the presence of SNPs associated with BZ-resistance and the ability of eggs to hatch without a chill stimulus. Exploring the current control measures in use throughout the UK, provided quantitative information on farm management, anthelmintic usage and parasite control as well as qualitative information on farmer's perceptions of disease on their farm. The unique insight into perceptions of disease severity, timings and changes over time together with parasite control plans highlighted knowledge gaps throughout the country which could be addressed in the future using targeted knowledge exchange programs. Information collected by the questionnaire was also used to populate the GLMM models discussed in chapters 6 and 7 with additional environmental data and experimental results from the genotyping and hatching experiments. The analysis discussed in this thesis provides an indication of factors which may be responsible for the changes observed in this species,

the potential risk factors highlighted could form the basis of future study to verify the associations estimated here and measure the impact of each factor with a view to developing industry recommendations.

The main risk factor for the presence of SNPs associated with BZ-resistance in *N. battus* was the lack of effective quarantine of new and returning stock. The importance of quarantine has been recognised for a long time (Coles and Roush, 1992; Falzon et al., 2014; Leathwick, 2004) and clear guidelines have been created to promote 'best practice' by farmers. Despite the tools being freely available, uptake remains low; a recent survey of UK and Irish sheep farmers found that although quarantine of incoming stock was being implemented on almost half of the farms tested, only 3% used a suitable strategy capable of preventing the introduction of resistant nematodes (Morgan et al., 2012). Effective quarantine is a simple message to relay to the agricultural community, protecting not only against the transmission of BZ-resistant *N. battus* but all infectious diseases of livestock. Recent research into the uptake of recommendations by farmers found that one of the key factors was the confirmation of anthelmintic resistance on their farm, suggesting that action is more likely to be taken after an event such as significant production loss due to drug failure than before (Jack et al., 2017). Perhaps a focus on changing farmer perceptions of industry recommendations and developing novel methods of promoting research outputs, such as integrating animal health advice into on-farm technology or greater use of demonstration farms, would be beneficial.

Recently, terms such as "smart farming" have frequently been discussed, suggesting that the future of the livestock industry relies upon the development of sensors and decision support systems, i.e. computer software which provides management advice. Technology capable of identifying production limiting factors earlier than current strategies, triggering treatment and minimising production losses. Decision support tools for managing parasite risk would

undoubtedly be beneficial however, the findings discussed in this thesis also highlight fundamental issues which could be addressed without the development of new devices. A greater understanding of parasite biology and knowledge exchange on the basic principles and benefits of existing control strategies such as quarantine practices may provide a significant benefit in the control of *N. battus* in the future. Pen-side diagnostics capable of detecting and quantifying SNPs conferring BZ-resistance in *N. battus* isolates would be useful in diagnosing anthelmintic resistance and monitoring the potential increase in SNP frequency over time. However, DNA extraction from *N. battus* eggs without laboratory equipment remains unfeasible at present.

The results presented in this thesis show the BZ-resistant alleles present on a large number of farms but predominant in only a limited number of cases. If *N. battus* is thought of as having a low refugia i.e. the population is largely active at the same time and all exposed during whole-flock BZ treatments then it may have been expected that once present, BZ-resistant alleles would quickly expand within the population. However, this was not observed in the majority of the populations sampled, suggesting that additional factors may be influencing the development of resistance in this species. Given the high farm-level prevalence of F200Y in *N. battus* populations throughout the UK and the continued favour of BZ compounds for the control of *N. battus*, it could be hypothesised that F200Y would be likely to increase in both prevalence and frequency in the near future. However, it is difficult to predict a timescale for the development and spread of resistance in this species due to the lack of understanding on the presence, size and importance of refugia in *N. battus* and the correlation between SNP frequency and anthelmintic efficacy. It is also difficult to predict the impact of changes in epidemiology in this species towards a more year-round infection similar to trichostrongylid species. BZ-resistance is widespread in other *Nematodirus* species with a recent survey of New Zealand populations reporting <95% FECR in 95% and 40% of *N. spathiger* and *N. filicollis* populations tested based on faecal egg count reduction tests (Oliver

et al., 2016a). The changes observed could result in a change in selection pressure or refugia status and BZ-resistance could progress to more closely resemble that of *N. spathiger* and other trichostrongylids in the future.

Although analysis in chapter 7 suggested that the ability of eggs to hatch without prior chilling was not correlated with the presence of the BZ-resistant allele F200Y, it is unlikely that the epidemiology of *N. battus* and the emergence of resistance in this species are not linked in any way. For example, North West England was identified as a focal region of the F200Y resistant allele and populations in this region appeared to have a largely spring-to-spring transmission pattern. In this situation, the majority of the population would be exposed to BZ treatment each spring therefore likely providing a significant selection pressure for BZ-resistance. Whereas, populations with a significant proportion of eggs hatching outside the expected spring window may be less likely to develop BZ-resistance, or do so at a slower pace. Only a proportion of the population would be exposed to treatment at any one time, providing a greater refugia compared to typical spring infection and whole-flock treatments. Given the plasticity of *N. battus* egg hatching, the evolution of this species and structure of refugia are very difficult to predict. Table 8.1 summarises the potential levels of refugia which could be expected for *N. battus* given different climatic, hatching and management scenarios. Understanding the risk of BZ-resistance development therefore relies on a robust knowledge of the full lifecycle of this species, which factors control hatching behaviour and the development of anthelmintic resistance and how these elements interact. The lack of correlation between F200Y allele frequency and the proportion of eggs hatching without chilling may indicate that these phenomena are opposing evolutionary pathways for *N. battus*. Both potentially driven by the strong selection pressure provided by whole-flock BZ treatments administered to control this species. Due to the relative isolation of *N. battus* populations, in comparison to other GIN species, as a result of limited movement of young stock, opposing mechanisms could evolve at a regional or farm level. Both

anthelmintic resistance and variation in hatching behaviour could be seen as methods for maximising transmission to the following year.

Table 8.1. Potential levels of refugia for N. battus predicted from a number of climatic, management and hatching scenarios.

A description of the sources of unexposed L3 available to re-infect animals immediately post-treatment and those available the following spring to dilute any resistant individuals which survived treatment

Scenario	Sources of unexposed individuals available for immediate re-infection	Sources of unexposed individuals to dilute resistant eggs for the following spring hatch	Estimated level of refugia
Spring-hatching population	• Spring-hatch L3 on pasture (high number of L3 but fast decay)	• Adult sheep • Wildlife reservoirs • Eggs passed out pre-treatment	**Medium** – large population size so any resistant eggs would be diluted on pasture but immediate reinfection may be low as L3 decay quickly on pasture
Spring-hatching population under variable climatic conditions (interrupted hatch)	• Spring-hatch L3 on pasture • Eggs stalled from hatching during the peak	• Adult sheep • Wildlife reservoirs • Eggs passed out pre-treatment	**High** – proportion of eggs unexposed to treatment but ready to hatch if climatic conditions return to the hatching range
Variable hatching population which interbreed	• Spring-hatch L3 on pasture • Eggs hatching throughout the remaining grazing season	• Adult sheep • Wildlife reservoirs • Eggs passed out pre-treatment • Eggs passed out from infection later in the grazing season	**High** – large, interbreeding population of which only a proportion of individuals may be exposed to treatment at any one time
Variable hatching population which do not interbreed	• Spring-hatch L3 on pasture (small population)	• Adult sheep • Wildlife reservoirs • Eggs passed out pre-treatment	**Low** – several small, isolated populations which are not available for re-infection of treated animals

The hatching characteristics of *N. battus* eggs were found to be associated with several environmental factors, especially spring temperature range, as may be expected based on previous research (van Dijk and Morgan, 2008). However, lambing date was also found to be associated with egg hatching, suggesting that the requirement for a chill stimulus may vary throughout the year, possibly influenced by the development of immunity in the aging lamb. If immune development were the driving influence then eggs may be predisposed to hatch without chilling in years where lambs fail to mount a strong, protective immunity following

a low or interrupted spring hatch, increasing the likelihood of successful transmission later in the season. Developmental variation in parasites in response to host immune development has been previously documented, for example in *strongyloides* species where the parasite switches from asexual to sexually reproducing adult morphs in response to host immune development (Gemmill et al., 1997; Harvey et al., 2000). The influence of lamb age and/or immune status could be investigated by repeating the hatching experiments described in chapter 7 using eggs collected from experimental infections of animals of different ages and immune status to explore differences in the requirement for chilling in eggs. Conducting repeated hatch experiments throughout the grazing season on eggs recovered from both experimental and field infections would also explain whether the requirement for a chill stimulus varies over time, i.e. throughout the grazing season. Developing an accurate picture of the variability of hatching requirements in *N. battus* eggs and the factors which alter hatching is important for the design of future control strategies. The design of grazing strategies to localise eggs with similar expected hatch dates on different paddocks would theoretically allow farmers to avoid grazing paddocks during specific high-risk periods however, this would not be practical for commercial enterprises. The future of *N. battus* is uncertain, if epidemiology continues to increase in variability, low level infection may occur throughout the year, in which case the severe symptoms associated with acute infection may not be present. However, in the advent of increasing prevalence of clinical BZ-resistance and variable hatching resulting in acute infection, alternative control methods such as the development of a vaccine may be required.

The findings discussed in this thesis showcase the complexity and variation observed in *N. battus* populations in the UK. This parasite species cannot be effectively controlled using a 'one-fits all' approach any longer, with the launch of online risk maps and recent changes in industry recommendations beginning to reflect this. The interest and support from the livestock industry has been highly motivating throughout the project and will hopefully

continue as research progresses on this topic. The risk factors highlighted by the current analysis should now provide the basis for in-depth continued study of *N.* battus, as summarised in Table 8.2. The fascinating plasticity of this species is of great academic interest but it is also economically important to the livestock industry as a clear understanding of the drivers of anthelmintic resistance and epidemiology are key to the design of effective control strategies to minimise production losses and protect animal welfare.

Table 8.2. Summary of the key findings of the project and research ideas to further explore and/or implement industry recommendations based on the findings.

Finding	What it means	Potential action points
Prevalence results		
SNPs associated with BZ-resistance are present in UK *N. battus* populations at a low frequency	Anthelmintic resistance is currently at an early stage in this species	- The early stage of resistance in this species offers the opportunity to investigate the origin(s) of anthelmintic resistance
F200Y present in ¼ of farm populations tested	Despite the low resistant allele frequency overall, the widespread distribution of resistant alleles could develop into clinical drug failure in the future given the appropriate selection pressure	- Use the results of risk factor analysis to reduce the likelihood of further development and dissemination of resistance
Risk factors		
Quarantine	SNPs associated with BZ-resistance in *N. battus* are more likely to be introduced from another source than originate on farm	- Knowledge exchange programs highlighting the importance of effective quarantine - Practical, easy to follow guides promoting best practice quarantine
Set stocked grazing	Set stocked grazing of lambs was associated with higher F200Y allele frequency	- Further research to explore the impact of set-stocked grazing in isolation - Design of grazing strategies based on future findings
Co-grazing sheep and cattle	Mixed species grazing was associated with lower F200Y resistant allele frequency	- Further research to verify and quantify the impact of co-grazing on resistant allele frequency - Future findings may be disseminated to farmers by targeted KE

Finding	What it means	Potential action points
Hatching results		
The requirement for a chill was varied between populations with a trend towards a greater proportion of eggs in the North of the UK not requiring a chill stimulus	Current UK populations do not all conform to the 'spring-only' hatching pattern previously documented for this species	- Current parasite forecast models could be updated to include the additional risk of hatching without prior chilling
Risk factors for changing epidemiology		
Grazing strategy Rest fields Reseeding 'Away' grazing	Variable grazing introduces uncertainty in host availability for infection therefore favouring eggs hatching without prior chilling/a proportion of eggs hatching at different times of year to maximise transmission opportunities	- Further research would be required to assess the impact of each of these factors on the hatching requirements of *N. battus* eggs over time perhaps using long-term grazing trials to validate and quantify the impact of variable grazing strategies.
Peak lambing week	Lambing week may signify an association of either host age or immune development/status and could indicate that the requirement for chilling may vary throughout the grazing season.	- Repeat of hatching experiment using eggs collected from experimental infection of lambs of differing age and immune status would provide information on the role of these factors - Repeated hatching experiments using eggs collected from the same population throughout the grazing season to monitor whether hatching requirements vary through time

Appendix 1

Genomic DNA sequence of benzimidazole susceptible and resistant isolates of *N. battus*.

```
Nb_BZ-susceptible   CATGTTCTCGATGT-GGTTCGAAAGAGGCTGAAGGATGTGATTGTCTGCAGGTTAAATGATTGTGAATGTGCTGGCAACTGGAGAGGTTT 89
Nb_BZ-resistant     ACTGTTCTCGATGTGGTTCGAAAAGAGGCTGAAGGATGTGATTGTCTGCAGGTTAAATGATTGTGAATGTGCTGGCAACTGGAGAGGTTT 90
                      ************ * *   ************************************************************************

Nb_BZ-susceptible   GCAAAATTGTAGTTTTAGGGCTTTCAACTGACTCATTCTCTTGGAGGAGGCACAGGTTCTGGCATGGGCACCCTACTCATTTCTAAAATT 179
Nb_BZ-resistant     GCAAAATTGTAGTT-TAGGGCTTTCAACTGACTCATTCTCTTGGAGGAGGCACAGGTTCTGGCATGGGCACCCTACTCATTTCTAAAATT 179
                     ************** ************************************************************************

Nb_BZ-susceptible   CGTGAGGAGTACCCCGATAGGATTATGGCATCATTCTCTGTCGTTCCGTCGCCGAAGGTAGGTGTGGCCTATCAAAATCAAAAGCTCTTC 269
Nb_BZ-resistant     CGTGAGGAGTACCCCGATAGGATTATGGCATCATTCTCTGTCGTTCCGTCGCCGAAGGTAGGTGTGGCCTATCAAAATTAAAAGCTCTTC 269
                    ************************************************************************ ************

Nb_BZ-susceptible   GTTAAATGCCGAACTTTAACGTTAACATCGCAACTTTTCAAGGTATCTGACACCGTTGTTGAGCCGTACAATGCTACTCTCTCTGTTCAT 359
Nb_BZ-resistant     GTTAAATGCCGAACTTTAACGTTAACATCGCAACTTTTCAAGGTATCTGACACCGTTGTTGAGCCGTACAATGCTACTCTCTCTGTTCAT 359
                    ********************************************************************************************

Nb_BZ-susceptible   CAGTTGGTAGAAAACACAGATGAAACTTTCTGCATTGACAACGAAGCTTTGTACGACATCTGTTTCCGAACATTGAAACTCACAAATCCA 449
Nb_BZ-resistant     CAGTTGGTAGAAAACACAGATGAAACTTACTGCATTGACAACGAAGCTTTGTACGACATCTGTTTCCGAACATTGAAACTCACAAATCCA 449
                    *************************** ************************************************************

Nb_BZ-susceptible   ACGTACGGAGATCTGAACCATCTAGGTATGGTCTTCCTAAGAGGTTTTTTTGGCCCCTTA------------------------------ 539
Nb_BZ-resistant     ACGTACGGAGATCTGAACCATCTAGGTATGGTCTTCCTAAGAGGTTTTTTTGTCTCTTTAATATAGAATTTTTAGTGTCTGTTACAATG 539
                    ************************************************** * * ***

Nb_BZ-susceptible   ---------------------------------------------------------------------------------------- 509
Nb_BZ-resistant     TCGGGTGTCACGACATGCCTTCGTTTCCCTGGACAACTCAACGCTGACCTTCGGAAGTTGGCGGTGAACATGGTTCCATTCCCGCGCCTT 629
       566

Nb_BZ-susceptible   ---------------------------------------------------------------------------------------- 509
Nb_BZ-resistant     CACTTCTTCATGCCAGGCTTTGCACCGCTGTCTGCAAAGGGAGCTCAAGCGTACCGTGCATTGACGGTTGCTGAACTCACACAGCAGGCA 719

Nb_BZ-susceptible   ------------------------------------------------------------------------------------         509
Nb_BZ-resistant     AGTTTTGGTCCCAGCGCTAATTTCGTATTGATCACCAGTAGTGTTCAGATGTTCGATGCGAAGAATATGATGGCAGCGGGGG         801
```

Appendix 2

Barcode primer adapter sequences for MiSeq library preparation, Nextera XT index kit V2 set (Illumina, USA)

i7 Index Name	i7 Bases in Adapter
Reverse primer adapters	
N701	TCGCCTTA
N702	CTAGTACG
N703	TTCTGCCT
N704	GCTCAGGA
N705	AGGAGTCC
N706	CATGCCTA
N707	GTAGAGAG
N710	CAGCCTCG
N711	TGCCTCTT
N712	TCCTCTAC
N714	TCATGAGC
N715	CCTGAGAT
N716	TAGCGAGT
N718	GTAGCTCC
N719	TACTACGC
N720	AGGCTCCG
N721	GCAGCGTA
N722	CTGCGCAT
N723	GAGCGCTA
N724	CGCTCAGT
N726	GTCTTAGG
N727	ACTGATCG
N728	TAGCTGCA

N729	GACGTCGA
Forward primer adapters	
S502	CTCTCTAT
S503	TATCCTCT
S505	GTAAGGAG
S506	ACTGCATA
S507	AAGGAGTA
S508	CTAAGCCT
S510	CGTCTAAT
S511	TCTCTCCG
S513	TCGACTAG
S515	TTCTAGCT
S516	CCTAGAGT
S517	GCGTAAGA
S518	CTATTAAG
S520	AAGGCTAT
S521	GAGCCTTA
S522	TTATGCGA

Appendix 3

Farm interview structure questions

Tell me about your farming operation?

- Type of enterprise (organic/pedigree/commercial/small holding)
- Upland/lowland/hill/other
- Livestock kept – detail and provide rough numbers. (sheep (inc number of ewes/tups/lambs)/cattle/goats/other)
- How long have you farmed at the current address? Land passed down from previous generation?
- Sheep breed kept? Main lamb flock?
- Numbers - breeding stock/lambs per year.
- Finish lambs or sell as store, breed replacements or buy in

What experience have you got with Nematodirus on your farm?

- To what extent? Losses?
- Control – treatments now and historically
- Any changes in recent years?
- Autumn?

Can you tell me how you typically use the fields on your farm?

- Which fields do you graze young lambs on?
- What is the typical movement of animals around the farm?
- What are these fields used for after lambs are moved? Silage?
- Autumn usage?
- Hill/fell grazing?
- Silage?

- Permanent or rotational grazing?
- Co-graze? Cattle/goats?
- Rest fields?

Can you tell me about your pasture management?

- Re-seeding - How often? Animals drenched onto new lay pasture?
- Pasture type - Ryegrass/white clover?
- Soil type
- Fertilisers
- Drainage – any water-logged areas? Flooding events? Water courses through fields?
- Shade?

So in terms of management, can you run me through lambing , for example when do you aim to start lambing, if they're indoor, any treatments around that time…?

- Typical dates
- Indoor/outdoor
- Time of weaning
- Treatment of ewes
- Typical movements of animals between lambing and weaning

- Do you graze mixed age lambs together?
- What is the typical stocking density of lambs pre- and post-weaning/ewes?
- Supplementary feed for lambs? What/when
- Drinking water – natural water course?

- Animals frequently moved? Frequently brought into sheds? Groups mixed?

What is your policy on buying in animals?

- Which markets do you use/where do you buy replacement stock from?
- Quarantine?

- Do you graze animals away from this farm?
- Do you buy in store lambs for finishing? Which fields do they typically graze? Where from?
- Do you sell/send lambs to be finished on another farm? Where?
- Do you share tups or communal grazing?

Can you tell me about diseases/infections on your farm in the past?

- Coccidia/fluke/others – when/treatment

Can you talk me through your roundworm control strategy?

- Drenches used throughout season – when/what
- Historic usage? Specifically BZs
- Storage
- Calibration of drench gun/weighing equipment
- How do you calculate the dosage given? Weigh/estimate/heaviest/mean...etc.
- Do you ever withhold food before/after drenching? How long for?
- Selective drenching?
- How do you determine drench dates?
- Dose and move?
- Do you routinely treat ewes? When/what
- Anthelmintic resistance/treatment failures in strongyles to any class?
- Regular FEC?

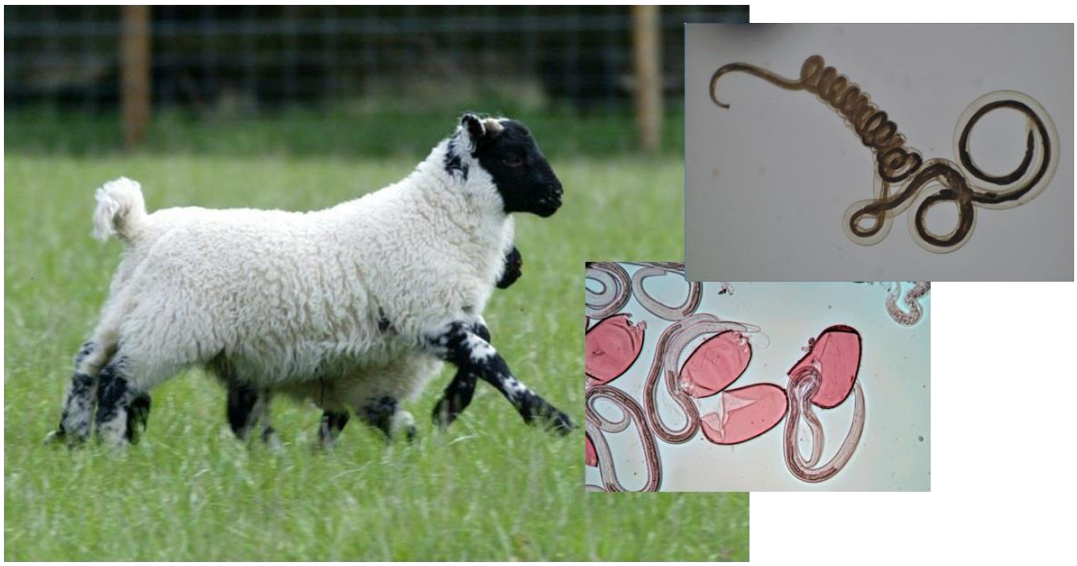
Do you administer mineral drenches? Any known deficiencies in the area?

Appendix 4

Farm management questionnaire

Thank you for participating in our survey into farm management strategies for controlling *Nematodirus battus*.

White drench resistance has recently been identified in *Nematodirus battus* populations in the UK. Resistance in this species is currently a low level however, has the potential to increase in the future. This questionnaire aims to gather information on farm management practices to allow analysis of potential risk factors which could be associated with the development and spread of white drench resistance in *Nematodirus*.
For further information on the project, please click here.



Farm details

* 1. Farm postcode

2. Email address

3. Type of enterprise

- [ ] Commercial
- [ ] Pedigree

Other (please specify)

4. Type of farm

- ( ) Lowland
- ( ) Upland
- ( ) Hill
- ( ) Other (please specify)

5. How long have you earned a living as a farmer from sheep at the current address?

- ( ) 0-5 years
- ( ) 5-20 years
- ( ) 20 years +

Farm demographics

6. Please provide rough numbers of livestock kept in the last 12 months

Breeding ewes	
Tups	
Lambs	
Cattle	
Goats	

7. Breed of lambs in main flock

8. Please specify typical dates for main lamb flock

Select all that apply

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Lambing	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Weaning	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

9. Which best describes your sheep enterprise?

- ◯ Breeder
- ◯ Feeder/finisher
- ◯ Both

10. Typical stocking densities

Heads per acre

Lambs and ewes pre-weaning	
Lambs post-weaning	

Nematodirus battus

Nematodirus battus typically infects young lambs in spring causing yellow/green scour and dehydration.

11. Have you ever had a *Nematodirus* on your farm in the last 5 years?

- ◯ Yes, severe symptoms observed in lambs (scouring and deaths)
- ◯ Yes, low level infection observed (scouring but no deaths)
- ◯ Yes, present but no signs of infection observed
- ◯ No, no signs of infection observed
- ◯ Don't know

Nematodirus battus: disease

12. Has scouring (or death) of lambs been **diagnosed** as *Nematodirus* infection?

- ☐ Yes, by faecal egg count
- ☐ Yes, by post mortem examination
- ☐ No
- ☐ Other (please specify)

13. When do your lambs typically show clinical signs of *Nematodirus*?

Select all that apply

- [ ] Spring (March-May)
- [ ] Summer (June-August)
- [ ] Autumn (September-November)
- [ ] Winter (December-February)

14. Do you see clinical symptoms of *Nematodirus* infection in breeding ewes and/or ewe lambs?

	Breeding ewes	Ewe lambs
Yes	○	○
No	○	○
Don't know	○	○

Nematodirus battus: treatment

15. Do you administer wormers to lambs to control *Nematodirus*?

- ○ Yes
- ○ No
- ○ Sometimes

Nematodirus battus: treatment

16. Have you used white wormers (benzimidazole; 1-BZ) to control ***Nematodirus*** in the past 12 months?

Select all which apply

- [ ] No
- [ ] Albacert (Downland)
- [ ] Albenil (Virbac)
- [ ] Albex (Chanelle AH)
- [ ] Allverm (Elanco)
- [ ] Benzimole (Mole Valley)
- [ ] Bovex (Chanelle)
- [ ] Endospec (Bimeda)
- [ ] Fenzol (Norbrook)
- [ ] Ovidown (Downland)
- [ ] Panacur (Norbrook)
- [ ] Parafend (Norbrook)
- [ ] Rycoben (Elanco)
- [ ] Tramazole (Tulivin/Denimex)
- [ ] Zerofen (chanelle AH)

Other (please specify)

Nematodirus battus: treatment

17. Have you used other types of wormer have you used to control ***Nematodirus*** in the last **12 months**?

- [ ] No
- [ ] Yellow drench (levamisole; 2-LV)
- [ ] Clear drench (macrocyclic lactone; 3-ML)
- [ ] Orange drench (Zolvix; 4-AD)
- [ ] Purple drench (Startect; 5-SI)

18. Which type of wormer have you predominantly used to control ***Nematodirus*** in the last **5 years**?

- [ ] None
- [ ] White drench (Benzimidazole; 1-BZ)
- [ ] Yellow drench (Levamisole; 2-LV)
- [ ] Clear drench (Macrocyclic lactone; 3-ML)
- [ ] Orange drench (Zolvix; 4-AD)
- [ ] Purple drench (Startect; 5-SI)

19. In your opinion, has ***Nematodirus*** infection on your farm changed in recent years?

Select all that apply

- [ ] Symptoms (e.g. diarrhoea) more severe
- [ ] Symptoms less severe
- [ ] Increased loss of stock
- [ ] Changes in timing of symptoms
- [ ] Treatment failures
- [ ] No change observed
- [ ] Other (please specify)

Nematodirus battus: treatment failure

20. How was treatment failure followed up?

Select all that apply

- [ ] Veterinary advice
- [ ] Faecal egg count reduction test
- [ ] Was not followed up
- [ ] Other (please specify)

Grazing management: pre-weaning

The lifecycle of *Nematodirus battus* may result in the development of high and low risk fields within each farming system. *Nematodirus* is transmitted from one year's lamb crop to the next, therefore grazing management of lambs may be important to better understanding the development of anthelmintic resistance in this parasite.

21. In the last 5 years, have 1-3 month old lambs grazed on the same field(s) each spring?

- ◯ Yes
- ◯ No

Grazing management: pre-weaning

22. Are fields routinely grazed by 1-3 month old lambs in spring grazed by sheep again in the autumn?

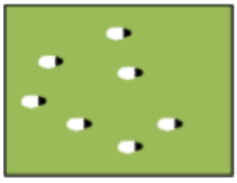
- [ ] Yes, mainly grazed by ewes
- [ ] Yes, mainly grazed by gimmers
- [ ] Yes, mainly grazed by lambs
- [ ] Yes, mainly grazed by tups
- [ ] No
- [ ] Sometimes

23. Have fields routinely grazed by 1-3 month old lambs been reseeded within the last 5 years?

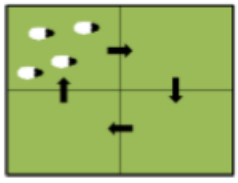
- ◯ Yes
- ◯ No

Grazing strategies

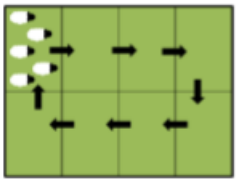
Set stocking
(Animals have unrestricted access over a wide area throughout the grazing season)



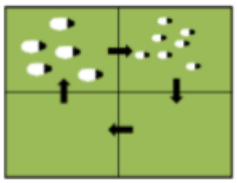
Rotational Grazing
(Stock is moved around a small number of fields based on grass growth or after a certain number of days)



Cellular/intensive grazing
(Livestock is moved frequently through a series of paddocks)



Leader-follower (forward creep) grazing
(Livestock move around a number of fields with older/less productive stock following younger/more productive stock)



24. Which grazing strategy (above) best describes your main strategy for grazing **lambs**?

- [ ] Set stocking
- [ ] Rotational grazing
- [ ] Cellular/intensive grazing
- [ ] Leader/follower grazing

Other (please specify)

25. When do you employ this grazing strategy?

- [ ] Throughout the year
- [ ] From weaning
- [ ] Other (please specify)

26. Do you routinely co-graze sheep with other animals?

Select all that apply

	Young beef cattle/store cattle	Adult beef cattle	Young dairy cattle	Adult dairy cattle	Goats	Horses
Permanently	☐	☐	☐	☐	☐	☐
Post-weaning	☐	☐	☐	☐	☐	☐
Sporadically throughout the year	☐	☐	☐	☐	☐	☐

27. Do you graze lambs on land separate from the main farm (away grazing)?

- ◯ Yes, all lambs
- ◯ Yes, a proportion of lambs each year
- ◯ Occasionally (not routinely each year)
- ◯ No
- ◯ Other (please specify)

28. When do you employ this grazing strategy?

- ◯ Throughout the year
- ◯ From weaning
- ◯ Other (please specify)

Grazing management

29. How many fields do you use in your grazing system?

30. Are fields grazed in the same order each year?

- ◯ Yes
- ◯ No

31. Approximately how many days do lambs graze each pasture?

32. Approximately how many days until lambs return to the first pasture?

33. Are lambs moved to different pasture immediately following wormer treatment?

- ◯ Yes
- ◯ No
- ◯ Sometimes

34. Do you routinely co-graze sheep with other animals?

Select all that apply

	Young beef cattle/store cattle	Adult beef cattle	Young dairy cattle	Adult dairy cattle	Goats	Horses
Permanently	☐	☐	☐	☐	☐	☐
Post-weaning	☐	☐	☐	☐	☐	☐
Sporadically throughout the year	☐	☐	☐	☐	☐	☐

35. Do you graze lambs on land separate from the main farm (away grazing)?

- ◯ Yes, all lambs
- ◯ Yes, a proportion of lambs each year
- ◯ Occasionally (not routinely each year)
- ◯ No
- ◯ Other (please specify)

Grazing management: rest fields

36. Do you rest fields?

- ◯ Yes
- ◯ No

Grazing management: rest fields

37. How long do you typically rest fields for?

Supplementary feed

38. Do you provide supplementary feed for lambs?

- ◯ Creep-feed pre-weaning
- ◯ Ad-lib feed post-weaning
- ◯ Feed at intervals post-weaning
- ◯ No supplementary feed provided
- ◯ Other (please specify)

Roundworm control

Anthelmintic treatments administered to control roundworms throughout the summer may have an impact upon the development of resistance in *Nematodirus*.

39. Which types of wormer have you used in the last 12 months to control **roundworms** in sheep?

Select all that apply

- [ ] I don't use wormers
- [ ] White drench (Benzimidazole; 1-BZ)
- [ ] Yellow drench (Levamisole; 2-LV)
- [ ] Clear drench (Macrocyclic lactone; 3-ML)
- [ ] Orange drench (Zolvix; 4-AD)
- [ ] Purple drench (Startect; 5-SI)

Roundworm control

40. How many wormer treatments do you administer each year?

	0	2	1	3	4	5	6+
Lambs	○	○	○	○	○	○	○
Ewes	○	○	○	○	○	○	○
Tups	○	○	○	○	○	○	○

41. How do you determine when to administer wormers?

Select all that apply

	Nematodirus control lambs	**Roundworm** infection lambs	**Roundworm** infection Ewes	**Roundworm** infection Tups
Symptoms of ill thrift (eg. scouring in lambs)	☐	☐	☐	☐
Faecal egg count	☐	☐	☐	☐
Weightgain	☐	☐	☐	☐
Following a vet plan	☐	☐	☐	☐
On risk map recommendation (e.g. NADIS or SCOPS)	☐	☐	☐	☐
Treatment administered at the same time each year	☐	☐	☐	☐
Pre-tupping	☐	☐	☐	☐
Pre/post-Lambing	☐	☐	☐	☐

Fluke control

Several white drench (benzimidazole; BZ-1) products are commonly used for the control of liver fluke. The behaviour of *nematodirus* appears to be changing with an increased incidence of infection and disease in autumn. Administration of benzimidazole products to control liver fluke may have an impact upon *Nematodirus* present in autumn.

42. Do you treat sheep for fluke?

- ◯ Yes
- ◯ No

Fluke control

43. Have you used any of the following white drenches to control liver fluke in the past 5 years?

Select all that apply

- ☐ Albacert (Downland)
- ☐ Albenil (Virbac)
- ☐ Albex (Chanelle)
- ☐ Allverm (Elanco)
- ☐ Benzimole (Mole Valley)
- ☐ Endospec (Bimeda)
- ☐ Ovidown (Downland)
- ☐ Rycoben (Elanco)
- ☐ Tramazole (Tulivin/Denimex)
- ☐ Don't know

Quarantine

44. Do you quarantine treat animals brought onto farm?

- ◯ Yes, all animals brought onto farm (including store lambs, replacement breeding stock and returning stock)
- ◯ Yes, replacement breeding stock only
- ◯ Sometimes
- ◯ No, never
- ◯ Other (please specify)

Quarantine

45. Which type of wormer do you use during quarantine?

Select all that apply

- [ ] White drench (Benzimidazole; 1-BZ)
- [ ] Yellow drench (Levamisole; 2-LV)
- [ ] Clear drench (Macrocyclic lactone; 3-ML)
- [ ] Orange drench (Zolvix; 4-AD)
- [ ] Purple drench (Startect; 5-SI)

46. Do you keep quarantine animals separate from other stock?

- ○ No
- ◯ Yes (please specify for how long)

End of survey

Thank you for completing our survey on the farm management of *Nematodirus.*

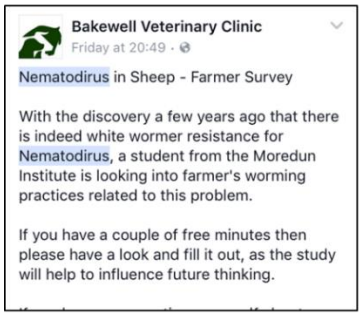
The information gathered will be used to assess potential risk factors associated with the development and spread of white drench resistance in *Nematodirus*. We hope to gain a greater knowledge of the mechanisms driving anthelmintic resistance in this roundworm. Knowledge gained in this project will be used to inform future 'best practice advice' aimed at reducing the impact and economic burden of anthelmintic resistance.

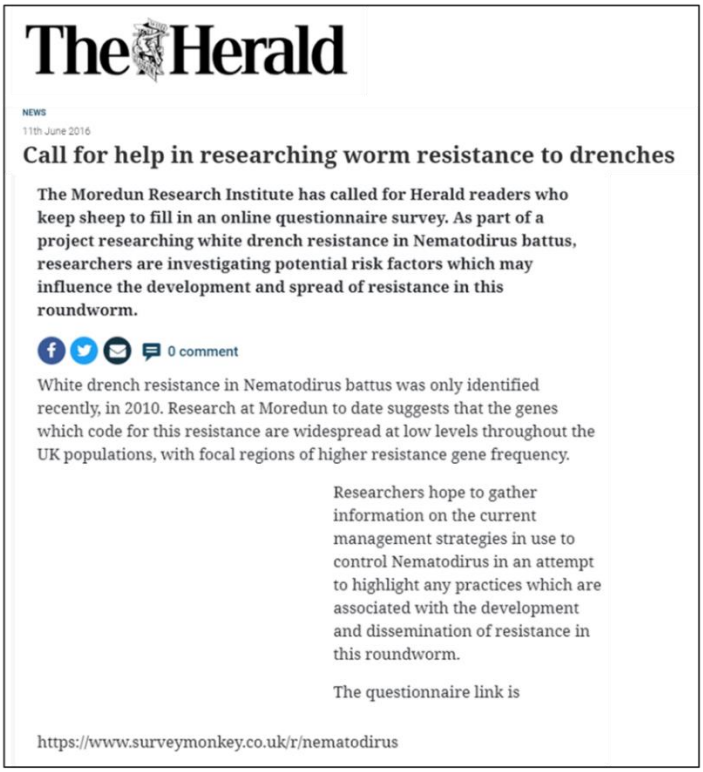
All information will be treated confidentially.

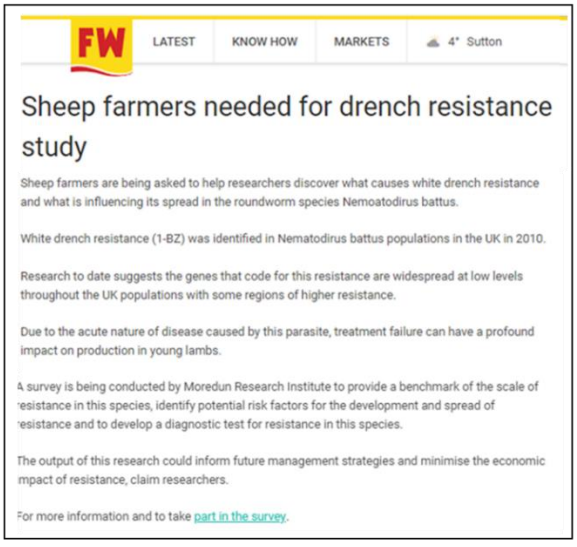
For further information on this project, please click here.

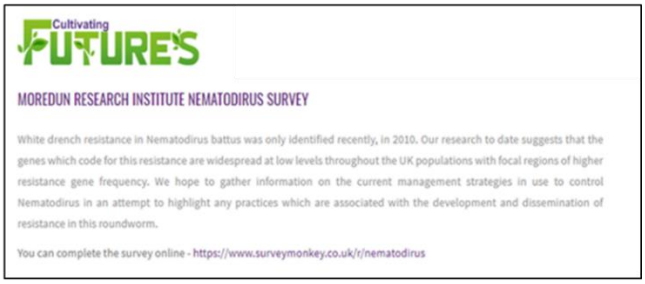
Appendix 5

Articles and social media posts used to disseminate the questionnaire









Appendix 6

Summary of questionnaire responses. (n= denotes the number of responses to each question).

Question	Response	All respondents		North Scotland		South Scotland		North east England		North west England		South central England		South east England		South west England		Wales	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Farming enterprise (n= 180)	Commercial	117	**65**	5	**56**	9	**69**	30	**81**	12	**75**	15	**71**	17	**57**	22	**56**	5	**42**
	Pedigree	29	**16**	2	**22**	1	**8**	3	**8**	2	**13**	2	**10**	8	**27**	6	**15**	4	**33**
	both	34	**19**	2	**22**	3	**23**	4	**11**	2	**13**	4	**19**	5	**17**	11	**28**	3	**25**
Time farming at current address (n= 183)	0-5 years	37	**20**	1	**11**	3	**23**	4	**10**	2	**13**	3	**13**	9	**30**	8	**21**	5	**42**
	5-20 years	53	**29**	4	**44**	3	**23**	10	**26**	6	**38**	6	**26**	10	**33**	12	**32**	1	**8**
	20+ years	93	**51**	4	**44**	7	**54**	25	**64**	8	**50**	14	**61**	11	**37**	18	**47**	6	**50**
Type of farm (n= 184)	Lowland	127	**69**	2	**25**	5	**38**	21	**54**	9	**60**	22	**96**	28	**90**	31	**78**	6	**50**
	Upland	40	**22**	4	**50**	4	**31**	14	**36**	1	**7**	0	**0**	3	**10**	9	**23**	5	**42**
	Hill	17	**9**	2	**25**	4	**31**	4	**10**	5	**33**	1	**4**	0	**0**	0	**0**	1	**8**

Question	Response	All respondents		North Scotland		South Scotland		North east England		North west England		South central England		South east England		South west England		Wales	
Sheep enterprise (n= 167)	Breeder	42	**25**	2	**25**	2	**17**	8	**25**	1	**7**	6	**29**	8	**29**	10	**26**	3	**33**
	Finisher	18	**11**	1	**13**	0	**0**	4	**13**	2	**14**	1	**5**	3	**11**	6	**15**	1	**11**
	Both	107	**64**	5	**63**	10	**83**	20	**63**	11	**79**	14	**67**	17	**61**	23	**59**	5	**56**

Questions relating to farmer's perceptions of *N. battus* on farm.

Question	Response	All respondents		North Scotland		South Scotland		North east England		North west England		South central England		South east England		South west England		Wales	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
***N. battus* symptoms on farm**	Lamb losses	24	**14**	1	**13**	5	**38**	3	**9**	4	**29**	1	**5**	3	**10**	6	**15**	1	**10**
	Scouring	73	**42**	3	**38**	4	**31**	15	**45**	7	**50**	11	**50**	9	**31**	18	**45**	4	**40**
(last 5 yrs)	Sub-clinical	35	**20**	1	**13**	2	**15**	2	**6**	2	**14**	8	**36**	6	**21**	9	**23**	5	**50**
(n= 171)	Not present	40	**23**	3	**38**	2	**15**	13	**39**	1	**7**	2	**9**	11	**38**	7	**18**	0	**0**
Diagnosis of *N. battus*	FEC	64	**52**	2	**50**	6	**60**	9	**45**	3	**23**	15	**79**	5	**29**	18	**62**	5	**63**
	Post-mortem	10	**8**	1	**25**	0	**0**	3	**15**	4	**31**	0	**0**	1	**6**	0	**0**	1	**13**
(n= 122)	None	48	**39**	1	**25**	4	**40**	8	**40**	6	**46**	4	**21**	11	**65**	11	**38**	2	**25**
Timing of clinical signs	Spring (Mar–May)	83	**55**	2	**50**	7	**58**	14	**50**	10	**67**	12	**57**	8	**44**	22	**54**	6	**67**
	Summer (Jun/Aug)	49	**33**	2	**50**	4	**33**	12	**43**	5	**33**	6	**29**	8	**44**	9	**22**	3	**33**
(n= 117)	Autumn (Sep-Nov)	13	**9**	0	**0**	1	**8**	2	**7**	0	**0**	2	**10**	1	**6**	7	**17**	0	**0**
	Winter (Dec-Feb)	5	**3**	0	**0**	0	**0**	0	**0**	0	**0**	1	**5**	1	**6**	3	**7**	0	**0**

Question	Response	All respondents		North Scotland		South Scotland		North east England		North west England		South central England		South east England		South west England		Wales	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
***N. battus* symptoms** (n=70)	Breeding ewes	21	**29**	1	**33**	1	**25**	6	**40**	2	**29**	2	**18**	2	**29**	6	**30**	1	**25**
	Ewe lambs	52	**71**	2	**67**	3	**75**	9	**60**	5	**71**	9	**82**	5	**71**	14	**70**	3	**75**
Changes in *N. battus* (n=118)	Symptoms more severe	10	**8**	0	**0**	1	**10**	3	**13**	4	**24**	1	**5**	0	**0**	0	**0**	0	**0**
	Symptoms less severe	17	**13**	1	**17**	2	**20**	4	**17**	2	**12**	0	**0**	4	**25**	4	**15**	0	**0**
	Increased loss of stock	4	**3**	1	**17**	0	**0**	1	**4**	1	**6**	1	**5**	0	**0**	0	**0**	0	**0**
	Time of year	16	**13**	0	**0**	0	**0**	3	**13**	0	**0**	4	**20**	3	**19**	2	**7**	3	**50**
	Treatment failures	5	**4**	1	**17**	0	**0**	0	**0**	3	**18**	1	**5**	0	**0**	0	**0**	0	**0**
	No change	76	**59**	3	**50**	7	**70**	12	**52**	7	**41**	13	**65**	9	**56**	21	**78**	3	**50**

Questions relating to grazing practices.

Question	Response	All respondents		North Scotland		South Scotland		North east England		North west England		South central England		South east England		South west England		Wales	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Grazing strategy for lambs (n= 168)	Set stock	64	38	5	63	8	62	11	33	9	64	6	29	13	46	6	15	4	44
	Rotational	86	51	3	38	5	38	17	52	4	29	14	67	10	36	28	72	4	44
	Cellular	10	6	0	0	0	0	1	3	0	0	0	0	3	11	4	10	1	11
	Leader/ follower	9	5	0	0	0	0	4	12	1	7	1	5	2	7	1	3	0	0
Reseeding of 'high risk' fields (n= 144)	Yes	31	22	2	29	4	36	4	14	3	23	2	12	4	16	9	27	2	29
	No	113	78	5	71	7	64	25	86	10	77	15	88	21	84	24	73	5	71
Repeated grazing of 'high risk' fields (n=164)	Yes	145	88	7	88	11	85	27	87	14	100	17	81	25	89	34	89	7	88
	No	20	12	1	13	2	15	4	13	0	0	4	19	3	11	4	11	1	13

Question	Response	All respondents		North Scotland		South Scotland		North east England		North west England		South central England		South east England		South west England		Wales	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Autumn grazing of 'high risk' fields (n= 119)	Ewe	119	60	6	75	8	50	24	62	13	59	14	56	18	55	28	67	6	60
	Gimmer	18	9	1	13	3	19	4	10	2	9	2	8	4	12	1	2	1	10
	Lamb	38	19	0	0	4	25	9	23	5	23	4	16	6	18	9	21	1	10
	Ram	4	2	0	0	0	0	0	0	2	9	1	4	0	0	0	0	1	10
	no	3	2	1	13	0	0	0	0	0	0	0	0	2	6	0	0	0	0
	sometimes	15	8	0	0	1	6	2	5	0	0	4	16	3	9	4	10	1	10
Away grazing (n= 163)	no	83	51	4	50	8	62	14	44	5	36	10	50	17	61	18	49	7	70
	occasionally	29	18	1	13	3	23	5	16	3	21	3	15	2	7	10	27	1	10
	proportion	41	25	3	38	1	8	10	31	5	36	7	35	8	29	5	14	2	20
	all	10	6	0	0	1	8	3	9	1	7	0	0	1	4	4	11	0	0

Question	Response	All respondents		North Scotland		South Scotland		North east England		North west England		South central England		South east England		South west England		Wales	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Rest fields (n= 162)	no	39	24	1	13	3	23	5	16	6	43	7	37	6	21	9	24	1	13
	yes	124	76	7	88	10	77	27	84	8	57	12	63	22	79	28	76	7	88
Supplementary feeding (n= 158)	Creep feed	49	31	1	13	1	8	6	19	6	46	6	32	11	41	12	34	4	50
	Post-weaning	10	6	2	25	0	0	3	9	1	8	0	0	0	0	4	11	0	0
	Intervals post-weaning	25	16	0	0	2	15	6	19	3	23	1	5	3	11	8	23	0	0
	None	75	47	5	63	10	77	17	53	3	23	12	63	13	48	11	31	4	50
Co-grazing lambs with other stock (n= 98)	Permanent	20	17	1	17	1	14	4	31	3	14	4	21	2	8	3	18	1	25
	Post-weaning	10	9	1	17	0	0	4	31	0	0	3	16	1	4	1	6	0	0
	Sporadic	86	74	4	67	6	86	5	38	19	86	12	63	23	88	13	76	3	75

Questions relating to anthelmintic treatment.

Question	Response	All responders		North Scotland		South Scotland		North east England		North west England		South central England		South east England		South west England		Wales	
		n	%	**n**	%	**n**	%	**n**	%	**n**	%	**n**	%	**n**	%	**n**	%	**n**	%
Method used to determine the timing of anthelmintic treatment (n= 149)	Ill thrift	87	**59**	3	**50**	8	**62**	17	**55**	5	**46**	12	**57**	18	**67**	18	**51**	6	**67**
	FEC	92	**63**	1	**17**	7	**54**	16	**52**	3	**27**	17	**81**	12	**44**	28	**80**	6	**67**
	Weightgain	28	**19**	3	**50**	1	**8**	6	**19**	0	**0**	7	**33**	5	**19**	7	**20**	1	**11**
	Vet plan	29	**20**	3	**50**	3	**23**	6	**19**	2	**18**	5	**24**	5	**19**	5	**14**	0	**0**
	Risk map	48	**33**	2	**33**	1	**8**	9	**29**	5	**46**	7	**33**	11	**41**	9	**26**	4	**44**
	Mating	69	**47**	5	**83**	5	**39**	19	**61**	2	**18**	9	**43**	12	**44**	13	**37**	4	**44**
	Lambing	86	**59**	4	**67**	4	**31**	16	**52**	7	**64**	12	**57**	17	**63**	21	**60**	5	**56**
	Yearly	50	**34**	3	**50**	6	**46**	12	**39**	7	**64**	5	**24**	8	**30**	7	**20**	2	**22**

Question	Response	All responders		North Scotland		South Scotland		North east England		North west England		South central England		South east England		South west England		Wales	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
1-BZ products used to control *N. battus* within the past year (n= 104)	Albacert	6	**6**	0	**0**	3	**43**	1	**6**	0	**0**	0	**0**	0	**0**	2	**7**	0	**0**
	Albenil	11	**11**	0	**0**	0	**0**	3	**18**	0	**0**	4	**25**	3	**20**	1	**3**	0	**0**
	Albex	22	**21**	1	**33**	1	**14**	7	**41**	2	**33**	1	**6**	5	**33**	1	**3**	4	**50**
	Allverm	8	**8**	1	**33**	0	**0**	0	**0**	0	**0**	4	**25**	1	**7**	0	**0**	2	**25**
	Benzimole	17	**16**	0	**0**	0	**0**	0	**0**	0	**0**	3	**19**	4	**27**	10	**34**	0	**0**
	Endospec	28	**27**	0	**0**	3	**43**	6	**35**	2	**33**	5	**31**	3	**20**	9	**31**	0	**0**
	Panacur	5	**5**	1	**33**	1	**14**	1	**6**	1	**17**	0	**0**	0	**0**	1	**3**	0	**0**
	Parafend	3	**3**	0	**0**	0	**0**	1	**6**	0	**0**	0	**0**	0	**0**	0	**0**	1	**13**
	Rycoben	32	**31**	2	**67**	2	**29**	4	**24**	2	**33**	3	**19**	3	**20**	14	**48**	2	**25**

Question	Response	All responders		North Scotland		South Scotland		North east England		North west England		South central England		South east England		South west England		Wales	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Anthelmintic classes used to control *N. battus* within the past year (n= 119)	1-BZ	104	**87**	3	**75**	8	**80**	18	**90**	6	**50**	16	**89**	15	**100**	29	**97**	8	**100**
	2-LV	25	**21**	0	**0**	2	**17**	7	**18**	6	**38**	5	**23**	4	**13**	2	**5**	1	**8**
	3-ML	35	**29**	2	**25**	5	**42**	10	**26**	4	**25**	2	**9**	5	**16**	4	**10**	1	**8**
	4-AD	6	**5**	0	**0**	0	**0**	0	**0**	0	**0**	3	**14**	0	**0**	3	**8**	0	**0**
	5-SI	1	**1**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	1	**3**	0	**0**	0	**0**
Anthelmintic classes used to control *N. battus* within the past five years (n= 119)	1-BZ	102	**54**	3	**38**	9	**75**	16	**42**	8	**50**	17	**77**	14	**45**	25	**63**	8	**67**
	2-LV	12	**6**	0	**0**	1	**8**	0	**0**	4	**25**	2	**9**	4	**13**	1	**3**	0	**0**
	3-ML	22	**12**	1	**13**	4	**33**	7	**18**	2	**13**	1	**5**	2	**6**	5	**13**	0	**0**
	4-AD	3	**2**	0	**0**	0	**0**	0	**0**	0	**0**	1	**5**	0	**0**	2	**5**	0	**0**
	5-SI	2	**1**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	1	**3**	0	**0**	1	**8**

Question	Response	All responders		North Scotland		South Scotland		North east England		North west England		South central England		South east England		South west England		Wales	
		n	%	**n**	%	**n**	%	**n**	%	**n**	%	**n**	%	**n**	%	**n**	%	**n**	%
Anthelmintic classes used to control GIN within the past five years (n= 159)	none	2	**1**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	2	**6**	0	**0**	0	**0**
	1-BZ	82	**52**	6	**75**	8	**67**	19	**50**	9	**56**	7	**32**	15	**48**	12	**30**	4	**36**
	2-LV	67	**42**	2	**25**	4	**33**	13	**34**	7	**44**	12	**55**	12	**39**	12	**30**	4	**36**
	3-ML	107	**68**	5	**63**	10	**83**	19	**50**	9	**56**	15	**68**	18	**58**	25	**63**	4	**36**
	4-AD	32	**20**	1	**13**	1	**8**	6	**16**	0	**0**	11	**50**	2	**6**	10	**25**	1	**9**
	5-SI	10	**6**	0	**0**	0	**0**	0	**0**	2	**13**	4	**18**	0	**0**	3	**8**	1	**9**
Fluke treatment (n= 159)	Yes	116	**73**	6	**75**	12	**100**	23	**77**	14	**100**	11	**55**	14	**50**	26	**72**	7	**88**
	No	43	**27**	2	**25**	0	**0**	7	**23**	0	**0**	9	**45**	14	**50**	10	**28**	1	**13**
1-BZ products used to control liver fluke within the past year (n=64)	Albacert	4	**6**	0	**0**	2	**29**	1	**7**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
	Albenil	9	**14**	0	**0**	2	**29**	1	**7**	0	**0**	1	**20**	4	**40**	0	**0**	1	**25**
	Albex	20	**31**	0	**0**	4	**57**	6	**40**	1	**17**	2	**40**	3	**30**	1	**9**	2	**50**
	Allverm	1	**2**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	1	**25**

Question	Response	All responders		North Scotland		South Scotland		North east England		North west England		South central England		South east England		South west England		Wales	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
1-BZ products used to control liver fluke within the past year (continued)	Benzimole	10	**16**	0	**0**	0	**0**	0	**0**	0	**0**	1	**20**	5	**50**	4	**36**	0	**0**
	Endospec	22	**34**	0	**0**	2	**29**	5	**33**	4	**67**	4	**80**	3	**30**	4	**36**	0	**0**
	Ovidown	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
	Rycoben	23	**36**	0	**0**	3	**43**	5	**33**	1	**17**	1	**20**	2	**20**	7	**64**	1	**25**
	Tramazole	3	**5**	0	**0**	0	**0**	1	**7**	0	**0**	0	**0**	1	**10**	0	**0**	0	**0**
Quarantine (n= 151)	No	10	**7**	1	**13**	0	**0**	4	**13**	2	**14**	0	**0**	0	**0**	3	**9**	0	**0**
	All	83	**55**	6	**75**	5	**42**	14	**47**	3	**21**	10	**63**	16	**62**	21	**62**	7	**88**
	Breeding stock only	34	**22**	1	**13**	3	**25**	6	**20**	5	**36**	6	**38**	5	**19**	5	**15**	1	**13**
	Sometimes	25	**16**	0	**0**	4	**33**	6	**20**	4	**29**	0	**0**	5	**19**	5	**15**	0	**0**

Question	Response	All responders		North Scotland		South Scotland		North east England		North west England		South central England		South east England		South west England		Wales	
		n	%	**n**	%	**n**	%	**n**	%	**n**	%	**n**	%	**n**	%	**n**	%	**n**	%
Quarantine treatment (n= 137)	1-BZ	29	**17**	2	**22**	5	**29**	8	**32**	4	**31**	1	**4**	4	**12**	2	**6**	2	**17**
	2-LV	21	**12**	2	**22**	3	**18**	0	**0**	2	**15**	1	**4**	7	**21**	2	**6**	2	**17**
	3-ML	63	**36**	3	**33**	6	**35**	11	**44**	3	**23**	8	**35**	13	**38**	13	**36**	3	**25**
	4-AD	52	**30**	1	**11**	3	**18**	6	**24**	2	**15**	10	**43**	9	**26**	17	**47**	4	**33**
	5-SI	10	**6**	1	**11**	0	**0**	0	**0**	2	**15**	3	**13**	1	**3**	2	**6**	1	**8**

Continuous variables.

Question		All respondents		North Scotland		South Scotland		North east England		North west England		South central England		South east England		South west England		Wales	
		n	mean ±SEM	n	mean ±SEM	n	mean ±SEM	n	mean ±SEM	n	mean ±SEM	n	mean ±SEM	n	mean ±SEM	n	mean ±SEM	n	mean ±SEM
Livestock kept (n= 166)	Ewes	166	532.5 ±78.5	8	472.8 ±198.2	13	797.6 ±135.8	30	526.0 ±107.5	14	1279.6 ±754.1	21	679.9 ±132.8	28	385.5 ±177.6	39	291.4 ±52.8	9	414.4 ±161.8
	Rams	166	15.3 ±1.6	8	18.4 ±8.1	13	30.1 ±7.1	30	13.0 ±2.3	14	15.4 ±2.8	21	17.5 ±3.2	27	8.9 ±3.2	38	17.0 ±5.8	9	9.4 ±1.8
	lambs	166	762.6 ±96.6	8	437.6 ±143.0	13	922.5 ±181.1	28	781.0 ±150.1	14	1544.6 ±748.8	21	1133.8 ±225.5	28	727.5 ±293.5	39	417.4 ±78.5	9	558.4 ±353.3
	Cattle	95	133.3 ±17.8	4	62.3 ±25.0	7	330.1 ±126.7	19	127.3 ±33.1	8	109.1 ±40.5	11	140.6 ±45.4	20	148.8 ±49.1	18	85.9 ±15.6	6	106.7 ±60.6
	Goats	5	55.6 ±36.8	1	1.0 ±0.0	1	40.0 ±0.0	1	7.0 ±0.0	0	0.0 ±0.0	1	30.0 ±0.0	0	0.0 ±0.0	1	200.0 ±0.0	0	0.0 ±0.0
Peak month (n= 167)	lambing	167	3.6 ±0.1	8	3.8 ±0.3	13	3.6 ±0.2	32	3.7 ±0.1	14	4.1 ±0.6	21	3.6 ±0.2	28	3.6 ±0.1	39	3.6 ±0.2	9	2.7 ±0.4
	weaning	155	7.4 ±0.1	7	7.6 ±0.2	11	8.0 ±0.2	27	7.9 ±0.1	14	7.2 ±0.4	18	6.8 ±0.3	28	7.5 ±0.2	39	7.3 ±0.2	9	6.5 ±0.3

Question		All respondents		North Scotland		South Scotland		North east England		North west England		South central England		South east England		South west England		Wales	
		n	mean ±SEM	n	mean ±SEM	n	mean ±SEM	n	mean ±SEM	n	mean ±SEM	n	mean ±SEM	n	mean ±SEM	n	mean ±SEM	n	mean ±SEM
Stocking density (n= 130)	pre-weaning	131	9.1 ±0.6	8	7.1 ±1.9	10	11.2 ±2.3	24	10.6 ±1.7	12	7.8 ±1.4	16	8.9 ±1.3	21	8.8 ±2.1	31	9.6 ±0.9	6	5.6 ±1.7
	post-weaning	125	9.5 ±0.6	8	7.6 ±2.3	10	12.7 ±3.1	24	11.3 ±1.5	10	9.0 ±2.1	16	8.8 ±0.9	20	8.6 ±1.9	29	9.7 ±0.8	5	5.5 ±2.1
Annual anthelmintic treatments (n= 152)	lambs	152	2.8 ±0.1	8	2.8 ±0.6	11	2.5 ±0.3	30	2.8 ±0.2	14	3.1 ±0.4	20	2.9 ±0.3	24	2.7 ±0.3	34	2.9 ±0.2	8	3.1 ±0.5
	Ewes	152	1.9 ±0.1	8	2.4 ±0.4	12	2.1 ±0.3	29	2.0 ±0.2	14	1.7 ±0.2	19	1.9 ±0.3	24	1.5 ±0.2	35	1.9 ±0.2	8	1.3 ±0.4
	Rams	136	2.0 ±0.1	8	2.5 ±0.4	10	2.0 ±0.3	24	1.8 ±0.2	14	1.9 ±0.2	16	2.0 ±0.3	23	1.9 ±0.2	31	2.0 ±0.2	7	1.6 ±0.3
Quarantine isolation time (n= 141)		141	25.6 ±3.8	7	20.1 ±4.7	11	21.2 ±3.3	27	18.3 ±3.3	12	14.0 ±2.9	17	49.1 ±20.2	25	19.7 ±1.6	31	35.8 ±12.1	8	17.3 ±4.6

9 References

Abaye, A.O., Allen, V.G., Fontenot, J.P., 1994. Influence of grazing cattle and sheep together and separately on animal performance and forage quality. Journal of Animal Science 72, 1013-1022.

Abbasi, I., Kirstein, O.D., Hailu, A., Warburg, A., 2016. Optimization of loop-mediated isothermal amplification (LAMP) assays for the detection of Leishmania DNA in human blood samples. Acta Tropical 162, 20-26.

Abbott, E.M., Parkins, J.J., Holmes, P.H., 1985. Influence of dietary protein on parasite establishment and pathogenesis in Finn Dorset and Scottish Blackface lambs given a single moderate infection of Haemonchus contortus. Research in Veterinary Science. 38, 6-13.

Abbott, K.A., Taylor, M., Stubbings, L.A., 2012. Susutainable worm control strategies for sheep 4th Edition.

Ai, L., Li, C., Elsheikha, H.M., Hong, S.J., Chen, J.X., Chen, S.H., Li, X., Cai, X.Q., Chen, M.X., Zhu, X.Q., 2010. Rapid identification and differentiation of Fasciola hepatica and Fasciola gigantica by a loop-mediated isothermal amplification (LAMP) assay. Veterinary Parasitology 174, 228-233.

Ali, D.N., Hennessy, D.R., 1993. The effect of feed intake on the rate of flow of digesta and the disposition and activity of oxfendazole in sheep. International Journal of Parasitology. 23, 477-484.

Annon. 2018. DIVA GIS [online] available at http://www.diva-gis.org/Data [acccessed 17th November 2018].

Arimatsu, Y., Kaewkes, S., Laha, T., Hong, S.J., Sripa, B., 2012. Rapid detection of Opisthorchis viverrini copro-DNA using loop-mediated isothermal amplification (LAMP). Parasitology International 61, 178-182.

Ash, C., Atkinson, H.J., 1984. Nematodirus battus: permeability changes, calcium binding, and phosphorylation of the eggshell during hatching. Experimental Parasitology 58, 27-40.

Ash, C.P.J., Atkinson, H.J., 1983. Evidence for a Temperature-Dependent Conversion of Lipid Reserves to Carbohydrate in Quiescent Eggs of the Nematode, Nematodirus-Battus. Comparative Biochemisrty and Physiology. 76, 603-610.

Avramenko, R.W., Redman, E.M., Lewis, R., Yazwinski, T.A., Wasmuth, J.D., Gilleard, J.S., 2015. Exploring the Gastrointestinal "Nemabiome": Deep Amplicon Sequencing to Quantify the Species Composition of Parasitic Nematode Communities. PLoS One 10, e0143559.

Avramenko, R.W., Redman, E.M., Melville, L., Bartley, Y., Wit, J., Queiroz, C., Bartley, D.J., Gilleard, J.S., 2018. Deep amplicon sequencing as a powerful new tool to screen for sequence polymorphisms associated with anthelmintic resistance in parasitic nematode populations. International Journal of Parasitology.

Badolo, A., Okado, K., Guelbeogo, W.M., Aonuma, H., Bando, H., Fukumoto, S., Sagnon, N., Kanuka, H., 2012. Development of an allele-specific, loop-mediated, isothermal amplification method (AS-LAMP) to detect the L1014F kdr-w mutation in Anopheles gambiae s. l. Malaria Journal 11, 227.

Bairden, K., Armour, J., 1987. *Nematodirus battus* infection in calves. Veterinary Record 121, 326-328.

Baker, D.W., 1939. Survival of worm parasite infection on New York State pastures. Cornell Veterinarian 29, 45-48.

Barger, I.A., Siale, K., Banks, D.J., Le Jambre, L.F., 1994. Rotational grazing for control of gastrointestinal nematodes of goats in a wet tropical environment. Veterinary Parasitology. 53, 109-116.
Barragry, T., 1984. Anthelmintics - A review. New Zealand Veterinary Journal 32, 161-164.
Barrere, V., Alvarez, L., Suarez, G., Ceballos, L., Moreno, L., Lanusse, C., Prichard, R.K., 2012. Relationship between increased albendazole systemic exposure and changes in single nucleotide polymorphisms on the beta-tubulin isotype 1 encoding gene in Haemonchus contortus. Veterinary Parasitology 186, 344-349.
Barrere, V., Falzon, L.C., Shakya, K.P., Menzies, P.I., Peregrine, A.S., Prichard, R.K., 2013. Assessment of benzimidazole resistance in Haemonchus contortus in sheep flocks in Ontario, Canada: comparison of detection methods for drug resistance. Veterinary Parasitology 198, 159-165.
Bartley, D.J., Jackson, E., Johnston, K., Coop, R.L., Mitchell, G.B.B., Sales, J., Jackson, F., 2003. A survey of anthelmintic resistant nematode parasites in Scottish sheep flocks. Veterinary Parasitology 117, 61-71.
Bentounsi, B., Attir, B., Meradi, S., Cabaret, J., 2007. Repeated treatment faecal egg counts to identify gastrointestinal nematode resistance in a context of low-level infection of sheep on farms in eastern Algeria. Veterinary Parasitology 144, 104-110.
Besier, R.B., 2008. Targeted treatment strategies for sustainable worm control in small ruminants. Tropical Biomedicine 25, 9-17.
Beveridge, I., Ellis, N.J., Riley, M.J., Brown, T.H., 1990. Prevalence of resistance in sheep nematode populations to benzimidazole and levamisole anthelmintics in the high rainfall areas of South Australia. Australian Veterinary Journal. 67, 413-415.
Black, W.J.M., 1959. A grassland management method of controlling nematodirus infestation. Journal of the British Grassland Society 14, 206-211.
Black, W.J.M., 1964. The development of a preventative routine against Nematodirus disease of lambs I. Administration of bephenium compounds at 21-day intervals. British Veterinary Journal 120, 301.
Boag, B., 1972. Helminth parasites of the wild rabbit Oryctolagus cuniculus (L.) in north east England. Journal of Helminthology 46, 73-78.
Boag, B., Thomas, R.J., 1975. Epidemiological studies on Nematodirus species in sheep. Research in Veterinary Science. 19, 263-268.
Borgsteede, F.H., 1983. Change in N battus epidemiology. Veterinary Record. 112, 554.
Borgsteede, F.H., Konig, C.D., 1979. [Nematodirus battus definitely established in the Netherlands (author's transl)]. Tijdschr Diergeneeskd 104, 825-828.
Brasil, B.S.A.F., Nunes, R.L., Bastianetto, E., Drummond, M.G., Carvalho, D.C., Leite, R.C., Molento, M.B., Oliveira, D.A.A., 2012. Genetic diversity patterns of Haemonchus placei and Haemonchus contortus populations isolated from domestic ruminants in Brazil. International Journal for Parasitology 42, 469-479.
British Deer Society, 2016. Deer distrobution study 2016 [online] available at https://www.bds.org.uk/index.php/research/deer-distribution-survey [accessed 12th December 2018].
Britt, D.P., 1982. Benzimidazole-resistant nematodes in Britain. Veterinary Record 110, 343-344.
Brown, H.D., Matzuk, A.R., Ilves, I.R., Peterson, L.H., Harris, S.A., Sarett, L.H., Egerton, J.R., Yakstis, J.J., Campbell, W.C., Cuckler, A.C., 1961. Antiparasitic drugs. IV. 2-(4'-thiazolyl)-benzimidazole, a new anthelmintic. Journal of the American Chemical Society 83, 1764-1765.
Bryant, C., Bennet, E.M., 1983. Observations on the fumerate reductase system in *Haemonchus contortus* and their relevance to anthelmintic resistance and to strain

variations of energy metabolism. Molecular and Biochemical Parasitology 7, 281-292.
Burgess, C.G., Bartley, Y., Redman, E., Skuce, P.J., Nath, M., Whitelaw, F., Tait, A., Gilleard, J.S., Jackson, F., 2012. A survey of the trichostrongylid nematode species present on UK sheep farms and associated anthelmintic control practices. Veterinary Parasitology 189, 299-307.
Calvete, C., Calavia, R., Ferrer, L.M., Ramos, J.J., Lacasta, D., Uriarte, J., 2012. Management and environmental factors related to benzimidazole resistance in sheep nematodes in Northeast Spain. Veterinary Parasitology 184, 193-203.
Cawthorne, R.J., Whitehead, J.D., 1983. Isolation of benzimidazole resistant strains of Ostertagia circumcincta from British sheep. Veterinary Record. 112, 274-277.
Centre for Sustainability and the Global Environment, 2018. Atlas of Biosphere [online] available at https://nelson.wisc.edu/sage/data-and-models/atlas/ [accessed 7th Deember 2018].
Chalmers, K., 1985. Detection of Benzimidazole Resistant Nematodirus-Spathiger. New Zealand Veterinary Journal 33, 53-53.
Chaudhry, F.R., Qayyum, M., Khan, M.F.U., Ahmad, T., Khanum, A., Shakir, M.R., Hussain, D., Miller, J.E., 2009. Peri-Parturient Rise in Faecal Nematode Egg Counts with Reference to Haemonchus contortus in Bulkhi Ewes in Northern Punjab, Pakistan. Pakistan Journal of Zoology 41, 437-443.
Chaudhry, U., Redman, E.M., Abbas, M., Muthusamy, R., Ashraf, K., Gilleard, J.S., 2015a. Genetic evidence for hybridisation between Haemonchus contortus and Haemonchus placei in natural field populations and its implications for interspecies transmission of anthelmintic resistance. International Journal of Parasitology 45, 149-159.
Chaudhry, U., Redman, E.M., Raman, M., Gilleard, J.S., 2015b. Genetic evidence for the spread of a benzimidazole resistance mutation across southern India from a single origin in the parasitic nematode Haemonchus contortus. International Journal of Parasitology 45, 721-728.
Chen, Z., Ye, W., Long, Z., Ding, D., Peng, H., Hou, X., Qiu, R., Xia, K., Tang, B., Jiang, H., 2015. Targeted Next-Generation Sequencing Revealed Novel Mutations in Chinese Ataxia Telangiectasia Patients: A Precision Medicine Perspective. PLoS One 10, e0139738.
Chintoan-Uta, C., Morgan, E.R., Skuce, P.J., Coles, G.C., 2014. Wild deer as potential vectors of anthelmintic-resistant abomasal nematodes between cattle and sheep farms. Proceedings. The Royal Society of Biological sciences 281, 20132985.
Coles, G.C., Bauer, C., Borgsteede, F.H.M., Geerts, S., Klei, T.R., Taylor, M.A., Waller, P.J., 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Veterinary Parasitology 44, 35-44.
Coles, G.C., Roush, R.T., 1992. Slowing the spread of anthelmintic resistant nematodes of sheep and goats in the United Kingdom. Veterinary Record 130, 505-510.
Coles, G.C., Simpkin, K.G., 1977. Resistance of nematode eggs to the ovicidal activity of benzimidazoles. Research in Veterinary Science 22, 386-387.
Conder, G.A., Campbell, W.C., 1995. Chemotherapy of nematode infections of veterinary importance, with special reference to drug-resistance. Advances in Parasitology 35, 1-84.
Conway, D.P., 1964. Variance in the Effectiveness of Thiabendazole against Haemonchus Contortus in Sheep. American Journal of Veterinary Research 25, 844-846.

Coop, R.L., Angus, K.W., Mapes, C.J., 1973. The effect of large doses of Nematodirus battus on the histology and biochemistry of the small intestine of lambs. International Journal of Parasitology 3, 349-361.
Coop, R.L., Holmes, P.H., 1996. Nutrition and parasite interaction. International Journal of Parasitology 26, 951-962.
Coop, R.L., Jackson, F., Jackson, E., 1991. Relative contribution of cattle to contamination of pasture with Nematodirus battus under an alternate grazing system of husbandry. Research in Veterinary Science 50, 211-215.
Coop, R.L., Jackson, F., Jackson, E., Fitzsimons, J., Lowman, B.G., 1988. Nematodirus infection in lambs on an alternate grazing system of husbandry. Research in Veterinary Science 45, 62-67.
Coop, R.L., Jackson, F., Wright, S.E., 1984. Observations on the role of calves in the transmission of *Nematodirus battus* in an alternate grazing system. Parasitology 89, R31-R31.
Coop, R.L., Kyriazakis, I., 1999. Nutrition-parasite interaction. Veterinary Parasitology 84, 187-204.
Coop, R.L., Kyriazakis, I., 2001. Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. Trends in Parasitology 17, 325-330.
Craven, J., Bjorn, H., Barnes, E.H., Henriksen, S.A., Nansen, P., 1999. A comparison of in vitro tests and a faecal egg count reduction test in detecting anthelmintic resistance in horse strongyles. Veterinary Parasitology 85, 49-59.
Crofton, H.D., Thomas, R.J., 1951. A New Species of Nematodirus in Sheep. Nature 168, 559-559.
Cudekova, P., Varady, M., Dolinska, M., Konigova, A., 2010. Phenotypic and genotypic characterisation of benzimidazole susceptible and resistant isolates of Haemonchus contortus. Veterinary Parasitology 172, 155-159.
Department for Environment, Food and Rural Affairs. 2014. Prevention of livestock disease [online] available at https://www.gov.uk/government/statistics/farm-practices-survey-october-2014-prevention-of-livestock-disease [accessed 12th December 2018].
Department for Environment, Food and Rural Affairs. 2017. Structure of the agricultural industry in England and the UK at June [online] available at https://www.gov.uk/government/statistical-data-sets/structure-of-the-agricultural-industry-in-england-and-the-uk-at-june [accessed 12th December 2018].
Diez-Banos, P., Pedreira, J., Sanchez-Andrade, R., Francisco, I., Suarez, J.L., Diaz, P., Panadero, R., Arias, M., Painceira, A., Paz-Silva, A., Morrondo, P., 2008. Field evaluation for anthelmintic-resistant ovine gastrointestinal nematodes by in vitro and in vivo assays. Journal of Parasitology 94, 925-928.
Diribe, O., North, S., Sawyer, J., Roberts, L., Fitzpatrick, N., La Ragione, R., 2014. Design and application of a loop-mediated isothermal amplification assay for the rapid detection of Staphylococcus pseudintermedius. Journal of Veterinary Diagnostic Investigation 26, 42-48.
Dobson, C., Bawden, R.J., 1974. Studies on the immunity of sheep to Oesophagostomum columbianum: effects of low-protein diet on resistance to infection and cellular reactions in the gut. Parasitology 69, 239-255.
Drame, P.M., Fink, D.L., Kamgno, J., Herrick, J.A., Nutman, T.B., 2014. Loop-mediated isothermal amplification for rapid and semiquantitative detection of Loa loa infection. Journal of Clinical Microbiology 52, 2071-2077.

Drudge, J.H., Szanto, J., Wyant, Z.N., Elam, G., 1964. Field Studies on Parasite Control in Sheep: Comparison of Thiabensazole, Ruelene, and Phenothiazine. American Journal of Veterinary Research 25, 1512-1518.

Duan, Y., Zhang, X., Ge, C., Wang, Y., Cao, J., Jia, X., Wang, J., Zhou, M., 2014. Development and application of loop-mediated isothermal amplification for detection of the F167Y mutation of carbendazim-resistant isolates in Fusarium graminearum. Nature Scientific Reports 4, 7094.

Dukes, J.P., King, D.P., Alexandersen, S., 2006. Novel reverse transcription loop-mediated isothermal amplification for rapid detection of foot-and-mouth disease virus. Archives of Virology 151, 1093-1106.

Dunn, A.M., 1965. The gastro-intestinal helminths of wild ruminants in Britain. I. Roe deer, Capreolus capreolus capreolus. Parasitology 55, 739-745.

Eddi, C., Caracostantogolo, J., Pena, M., Schapiro, J., Marangunich, L., Waller, P.J., Hansen, J.W., 1996. The prevalence of anthelmintic resistance in nematode parasites of sheep in southern Latin America: Argentina. Veterinary Parasitology 62, 189-197.

Edwards, T., Burke, P.A., Smalley, H.B., Gillies, L., Hobbs, G., 2014. Loop-Mediated Isothermal Amplification Test for Detection of Neisseria gonorrhoeae in Urine Samples and Tolerance of the Assay to the Presence of Urea. Journal of Clinical Microbiology 52, 2163-2165.

Elard, L., Humbert, J.F., 1999. Importance of the mutation of amino acid 200 of the isotype 1 beta-tubulin gene in the benzimidazole resistance of the small ruminant parasite Teladorsagia circumcincta. Parasitology Research 85, 452-456.

Elard, L., Sauve, C., Humbert, J.F., 1998. Fitness of benzimidazole-resistant and -susceptible worms of Teladorsagia circumcincta, a nematode parasite of small ruminants. Parasitology 117, 571-578.

England, R., Pettersson, M., 2005. Pyro Q-CpG (TM): quantitative analysis of methylation in multiple CpG sites by Pyrosequencing (R). Nature Methods 2, [online] available at https://www.nature.com/app_notes/nmeth/2005/050929/full/nmeth800.html [accessed 15th December 2018].

Enomoto, Y., Yoshikawa, T., Ihira, M., Akimoto, S., Miyake, F., Usui, C., Suga, S., Suzuki, K., Kawana, T., Nishiyama, Y., Asano, Y., 2005. Rapid diagnosis of herpes simplex virus infection by a loop-mediated isothermal amplification method. Journal of Clinical Microbiology 43, 951-955.

Esteban-Ballesteros, M., Rojo-Vazquez, F.A., Skuce, P.J., Melville, L., Gonzalez-Lanza, C., Martinez-Valladares, M., 2017. Quantification of resistant alleles in the beta-tubulin gene of field strains of gastrointestinal nematodes and their relation with the faecal egg count reduction test. BMC Veterinary Research 13, 71.

Falzon, L.C., O'Neill, T.J., Menzies, P.I., Peregrine, A.S., Jones-Bitton, A., vanLeeuwen, J., Mederos, A., 2014. A systematic review and meta-analysis of factors associated with anthelmintic resistance in sheep. Preventive Veterinary Medicine 117, 388-402.

Fan, Q., Xie, Z., Xie, L., Liu, J., Pang, Y., Deng, X., Xie, Z., Peng, Y., Wang, X., 2012. A reverse transcription loop-mediated isothermal amplification method for rapid detection of bovine viral diarrhea virus. Journal of Virological Methods 186, 43-48.

Fang, X., Chen, H., Yu, S., Jiang, X., Kong, J., 2011. Predicting viruses accurately by a multiplex microfluidic loop-mediated isothermal amplification chip. Analytical chemistry 83, 690-695.

Faruqi, A.F., Hosono, S., Driscoll, M.D., Dean, F.B., Alsmadi, O., Bandaru, R., Kumar, G., Grimwade, B., Zong, Q., Sun, Z., Du, Y., Kingsmore, S., Knott, T., Lasken, R.S., 2001. High-throughput genotyping of single nucleotide polymorphisms with rolling circle amplification. BMC Genomics 2, 4.

Ferrari, N., Cattadori, I. M., Rizzoli, A., Hudson, P. J., 2009. *Heligosomoides polygyrus* reduces infestation of *Ixodes ricinus* in free-living yellow-necked mice, *Apodemus flavicollis*. Parasitology 136, 305-316.

Fukuta, S., Mizukami, Y., Ishida, A., Kanbe, M., 2006. Development of loop-mediated isothermal amplification (LAMP)-based SNP markers for shelf-life in melon (Cucumis melo L.). Journal of Applied Genetics 47, 303-308.

Gadkar, V.J., Goldfarb, D.M., Gantt, S., Tilley, P.A.G., 2018. Real-time Detection and Monitoring of Loop Mediated Amplification (LAMP) Reaction Using Self-quenching and De-quenching Fluorogenic Probes. Nature Scientific Reports 8, 5548.

Gemmill, A.W., Viney, M.E., Read, A.F., 1997. Host Immune Status Determines Sexuality in a Parasitic Nematode. Evolution 51, 393-401.

Gethings, O.J., Rose, H., Mitchell, S., van Dijk, J., Morgan, E.R., 2015. Asynchrony in host and parasite phenology may decrease disease risk in livestock under climate warming: Nematodirus battus in lambs as a case study. Parasitology 142, 1306-1317.

Ghisi, M., Kaminsky, R., Maser, P., 2007. Phenotyping and genotyping of Haemonchus contortus isolates reveals a new putative candidate mutation for benzimidazole resistance in nematodes. Veterinary Parasitology 144, 313-320.

Gibson, T.E., 1963. Experiments on the epidemiology of nematodiriasis. Research in Veterinary Science 4, 11.

Gibson, T.E., Everett, G., 1973. Observations on the control of nematodiriasis and trichostrongylosis in sheep. Journal of Comparative Pathology 83, 125-132.

Gibson, T.E., Everett, G., 1981. Ecology of the Free Living Stages of Nematodirus-Battus. Research in Veterinary Science 31, 323-327.

Gilleard, J.S., Beech, R.N., 2007. Population genetics of anthelmintic resistance in parasitic nematodes. Parasitology 134, 1133-1147.

Glenn, T.C., 2011. Field guide to next-generation DNA sequencers. Molecular Ecology Resources 11, 759-769.

Good, B., Hanrahan, J.P., Crowley, B.A., Mulcahy, G., 2006. Texel sheep are more resistant to natural nematode challenge than Suffolk sheep based on faecal egg count and nematode burden. Veterinary Parasitology 136, 317-327.

Gray, C.M., Katamba, A., Narang, P., Giraldo, J., Zamudio, C., Joloba, M., Narang, R., Paramasivan, C.N., Hillemann, D., Nabeta, P., Amisano, D., Alland, D., Cobelens, F., Boehme, C.C., 2016. Feasibility and Operational Performance of Tuberculosis Detection by Loop-Mediated Isothermal Amplification Platform in Decentralized Settings: Results from a Multicenter Study. Journal of Clinical Microbiology 54, 1984-1991.

Gremer, J.R., Venable, D.L., 2014. Bet hedging in desert winter annual plants: optimal germination strategies in a variable environment. Ecology Letters 17, 380-387.

Grimshaw, W.T.R., Hunt, K.R., Hong, C., Coles, G.C., 1994. Detection of anthelmintic resistant nematodes in sheep in Southern England by a faecal egg count reduction test. Veterinary Record 135, 372-374.

Ha, B.K., Hussey, R.S., Boerma, H.R., 2007. Development of SNP assays for marker-assisted selection of two southern root-knot nematode resistance QTL in soybean. Crop Science 47, S73-S82.

Han, E.T., Watanabe, R., Sattabongkot, J., Khuntirat, B., Sirichaisinthop, J., Iriko, H., Jin, L., Takeo, S., Tsuboi, T., 2007. Detection of four Plasmodium species by genus- and species-specific loop-mediated isothermal amplification for clinical diagnosis. Journal of Clinical Microbiology 45, 2521-2528.

Hansen, T.V.A., Nejsum, P., Olsen, A., Thamsborg, S.M., 2013. Genetic variation in codons 167, 198 and 200 of the beta-tubulin gene in whipworms (Trichuris spp.) from a range of domestic animals and wildlife. Veterinary Parasitology 193, 141-149.

Harvey, S.C., Gemmill, A.W., Read, A.F., Viney, M.E., 2000. The control of morph development in the parasitic nematode Strongyloides ratti. Proceedings. The Royal Society of Biological sciences 267, 2057-2063.
Helle, O., 1969. The introduction of Nematodirus battus (Crofton and Thomas, 1951) into a new environment. Veterinary Record. 84, 157-160.
Hill, J., Beriwal, S., Chandra, I., Paul, V.K., Kapil, A., Singh, T., Wadowsky, R.M., Singh, V., Goyal, A., Jahnukainen, T., Johnson, J.R., Tarr, P.I., Vats, A., 2008. Loop-mediated isothermal amplification assay for rapid detection of common strains of Escherichia coli. Journal of Clinical Microbiology 46, 2800-2804.
Hoberg, E.P., 2005. Coevolution and biogeography among Nematodirinae (Nematoda : Trichostrongylina) Lagomorpha and Artiodactyla (Mammalia): Exploring determinants of history and structure for the northern fauna across the Holarctic. Journal of Parasitology 91, 358-369.
Hoberg, E.P., Zimmerman, G.L., Lichtenfels, J.R., 1986. First report of *Nematodirus battus* (Nematoda, Trichostrongyloidea) in North America - redescription and comparison to other species. Proceedings of the Helminthological Society of Washington 53, 80-88.
Hollands, R., 2018. Nematodirus battus infection in lambs. Veterinary Record 183, 267-268.
Hollands, R.D., 1984. Autumn nematodiriasis. Veterinary Record. 115, 526-527.
Hoste, H., Mallet, S., Fort, G., 1993. Histopathology of the small-intestinal mucosa in *Nematodirus spathiger* infection in rabbits. Journal of Helminthology 67, 139-144.
Hotson, I.K., Campbell, N.J., Smeal, M.G., 1970. Anthelmintic resistance in Trichostrongylus colubriformis. Austrialian Veterinary Journal 46, 356-360.
Hsieh, K., Patterson, A.S., Ferguson, B.S., Plaxco, K.W., Soh, H.T., 2012. Rapid, sensitive, and quantitative detection of pathogenic DNA at the point of care through microfluidic electrochemical quantitative loop-mediated isothermal amplification. Angewandte Chemie International Edition English 51, 4896-4900.
Hubert, J., Kerboeuf, D., 1985. Study of Gastrointestinal Strongylosis in A Sheep Flock on Permanent Pasture .2. Sheep Parasitism in 1978-1979. Annales de Recherches Veterinaires 16, 29-39.
Hughes, P.L., Dowling, A.F., Callinan, A.P., 2007. Resistance to macrocyclic lactone anthelmintics and associated risk factors on sheep farms in the lower North Island of New Zealand. New Zealand Veterinary Journal 55, 177-183.
Illumina, 2018. An introduction to next-generation sequencing technology [online] available at https://emea.illumina.com/content/dam/illumina-marketing/documents/products /illumina_sequencing_introduction.pdf [accessed 7th December 2018].
Iseki, H., Alhassan, A., Ohta, N., Thekisoe, O.M., Yokoyama, N., Inoue, N., Nambota, A., Yasuda, J., Igarashi, I., 2007. Development of a multiplex loop-mediated isothermal amplification (mLAMP) method for the simultaneous detection of bovine Babesia parasites. Journal of Microbiology Methods 71, 281-287.
Israf, D.A., Coop, R.L., Stevenson, L.M., Jones, D.G., Jackson, F., Jackson, E., Mackellar, A., Huntley, J.F., 1996. Dietary protein influences upon immunity to Nematodirus battus infection in lambs. Veterinary Parasitology 61, 273-286.
Israf, D.A., Jackson, F., Stevenson, L.M., Jones, D.G., Jackson, E., Huntley, J.F., Coop, R.L., 1997. Persistence of immunity to Nematodirus battus infection in lambs. Veterinary Parasitology 71, 39-52.
Jack, C., Hotchkiss, E., Sargison, N.D., Toma, L., Milne, C., Bartley, D.J., 2017. A quantitative analysis of attitudes and behaviours concerning sustainable parasite control practices from Scottish sheep farmers. Preventive Veterinary Medicine 139, 134-145.

Jackson, F., Christie, M., 1972. Quantitative recovery of floatable helminth eggs from 1 g. of ruminant faeces for counting followed by hatching for identification. Transactions of The Royal Society of Tropical Medicine and Hygine 66, 546.
Jackson, F., Coop, R.L., 2000. The development of anthelmintic resistance in sheep nematodes. Parasitology 120, S95-S107.
Jackson, F., Miller, J., 2006. Alternative approaches to control-Quo vadit? Veterinary Parasitology 139, 371-384.
Jackson, R.A., 1982. Possible *Nematodirus* resistance. Surveillance 9, 24-26.
Jagdale, G.B., Grewal, P.S., 2003. Acclimation of entomopathogenic nematodes to novel temperatures: trehalose accumulation and the acquisition of thermotolerance. International Journal of Parasitology 33, 145-152.
Jansen, J., 1973. Where does Nematodirus battus Crofton & Thomas, 1951, come from? Veterinary Record 92, 697-698.
Jeger, M.J., van Den Bosch, F., Madden, L.V., Holt, J., 1998. A model for analysing plant-virus transmission characteristics and epidemic development. Ima Journal of Mathematics Applied in Medicine and Biology 15, 1-18.
Jung, M.K., Wilder, I.B., Oakley, B.R., 1992. Amino acid alterations in the benA (beta-tubulin) gene of Aspergillus nidulans that confer benomyl resistance. Cell Motilility and the Cytoskeleton 22, 170-174.
Kambara, T., McFarlane, R.G., Abell, T.J., McAnulty, R.W., Sykes, A.R., 1993. The effect of age and dietary protein on immunity and resistance in lambs vaccinated with Trichostrongylus colubriformis. International Journal of Parasitology 23, 471-476.
Kaneko, H., Kawana, T., Fukushima, E., Suzutani, T., 2007. Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. Journal of Biochemical and Biophysical Methods 70, 499-501.
Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends in Parasitology 20, 477-481.
Karthik, K., Rathore, R., Thomas, P., Arun, T.R., Viswas, K.N., Agarwal, R.K., Manjunathachar, H.V., Dhama, K., 2014. Loop-mediated isothermal amplification (LAMP) test for specific and rapid detection of Brucella abortus in cattle. Vet Q 34, 174-179.
Kates, K.C., Turner, J.H., 1955. Observations on the life cycle of Nematodirus spathiger, a nematode parasitic in the intestine of sheep and other ruminants. American Journal of Veterinary Research 16, 105-115.
Keane, O.M., Keegan, J.D., Good, B., de Waal, T., Fanning, J., Gottstein, M., Casey, M., Hurley, C., Sheehan, M., 2014. High level of treatment failure with commonly used anthelmintics on Irish sheep farms. Irish Veterinary Journal 67, 16.
Kenyon, F., Greer, A.W., Coles, G.C., Cringoli, G., Papadopoulos, E., Cabaret, J., Berrag, B., Varady, M., van Wyk, J.A., Thomas, E., Vercruysse, J., Jackson, F., 2009. The role of targeted selective treatments in the development of refugia-based approaches to the control of gastrointestinal nematodes of small ruminants. Veterinary Parasitology 164, 3-11.
Kenyon, F., McBean, D., Greer, A.W., Burgess, C.G., Morrison, A.A., Bartley, D.J., Bartley, Y., Devin, L., Nath, M., Jackson, F., 2013. A comparative study of the effects of four treatment regimes on ivermectin efficacy, body weight and pasture contamination in lambs naturally infected with gastrointestinal nematodes in Scotland. International Journal of Parasitology Drugs Drug Resist. 3, 77-84.
Keymer, A., 1982. Density-dependent mechanisms in the regulation of intestinal helminth populations. Parasitology 84, 573-587.
Khan, M.G., Bhaskar, K.R., Salam, M.A., Akther, T., Pluschke, G., Mondal, D., 2012. Diagnostic accuracy of loop-mediated isothermal amplification (LAMP) for

detection of Leishmania DNA in buffy coat from visceral leishmaniasis patients. Parasites and Vectors 5, 280.
Khan, W.I., Collins, S.M., 2004. Immune-mediated alteration in gut physiology and its role in host defence in nematode infection. Parasite immunology 26, 319-326.
Kingsbury, P.A., 1953. Nematodirus infestation - a probable cause of losses amongst lambs. Veterinary Record 65, 167-169.
Kinsley, A.C., Patterson, G., VanderWaal, K.L., Craft, M.E., Perez, A.M., 2016. Parameter Values for Epidemiological Models of Foot-and-Mouth Disease in Swine. Frontiers in veterinary science 3, 44.
Knowles, S. C. L., Fenton, A., Petchey, O. L., Jones, T. R., Barber, R., Pedersen, A. B., 2013. Stability of within-host-parasite communities in a wild mammal system. Proceedings of the Royal Society of Biology 280, 20130598.
Koenraadt, H., Somerville, S.C., Jones, A.L., 1992. Characterization of Mutations in the Beta-Tubulin Gene of Benomyl-Resistant Field Strains of Venturia-Inaequalis and Other Plant Pathogenic Fungi. Phytopathology 82, 1348-1354.
Kotze, A.C., Cowling, K., Bagnall, N.H., Hines, B.M., Ruffell, A.P., Hunt, P.W., Coleman, G.T., 2012. Relative level of thiabendazole resistance associated with the E198A and F200Y SNPs in larvae of a multi-drug resistant isolate of Haemonchus contortus. International Journal for Parasitology: Drugs and Drug Resistance 2, 92-97.
Kurosaki, Y., Takada, A., Ebihara, H., Grolla, A., Kamo, N., Feldmann, H., Kawaoka, Y., Yasuda, J., 2007. Rapid and simple detection of Ebola virus by reverse transcription-loop-mediated isothermal amplification. Journal of Virological Methods 141, 78-83.
Kwa, M.S., Kooyman, F.N., Boersema, J.H., Roos, M.H., 1993. Effect of selection for benzimidazole resistance in Haemonchus contortus on beta-tubulin isotype 1 and isotype 2 genes. Biochemical and Biophysical Research Communications 191, 413-419.
Kwa, M.S., Veenstra, J.G., Roos, M.H., 1994. Benzimidazole resistance in Haemonchus contortus is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. Molecular and Biochemical Parasitology 63, 299-303.
Kwa, M.S., Veenstra, J.G., van Dijk, M., Roos, M.H., 1995. Beta-tubulin genes from the parasitic nematode Haemonchus contortus modulate drug resistance in Caenorhabditis elegans. Journal of Molecular Biology 246, 500-510.
Kyriazakis, I., Oldham, J.D., Coop, R.L., Jackson, F., 1994. The Effect of Subclinical Intestinal Nematode Infection on the Diet Selection of Growing Sheep. British Journal of Nutrition 72, 665-677.
Lacey, E., 1988. The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimadazoles. International Journal for Parasitology 18, 885-936.
Lacey, E., 1990. Mode of action of benzimidazoles. Parasitology Today 6, 112-115.
Lacey, E., Gill, J.H., 1994. Biochemistry of benzimidazole resistance. Acta Tropical 56, 245-262.
Larsen, J.W., Anderson, N., Vizard, A.L., 1999. The pathogenesis and control of diarrhoea and breech soiling in adult Merino sheep. International Journal of Parasitology 29, 893-902.
Lawrence, K.E., Rhodes, A.P., Jackson, R., Leathwick, D.M., Heuer, C., Pomroy, W.E., West, D.M., Waghorn, T.S., Moffat, J.R., 2006. Farm management practices associated with macrocyclic lactone resistance on sheep farms in New Zealand. New Zealand Veterinary Journal 54, 283-288.
Le Jambre, L.F., 1976. Egg hatch as an *in vitro* assay of thiabendazole resistance in nematodes. Veterinary Parasitology 2, 385-391.

Le Jambre, L.F., 1979. Effectiveness of anthelmintic treatments against levamisole-resistant Ostertagia. Australian Veterinary Journal 55, 65-67.
Le Jambre, L.F., Southcott, W.H., Dash, K.M., 1977. Resistance of selected lines of Ostertagia circumcincta to thiabendazole, morantel tartrate and levamisole. International Journal of Parasitology 7, 473-479.
Le Roux, C.A., Kubo, T., Grobbelaar, A.A., van Vuren, P.J., Weyer, J., Nel, L.H., Swanepoel, R., Morita, K., Paweska, J.T., 2009. Development and evaluation of a real-time reverse transcription-loop-mediated isothermal amplification assay for rapid detection of Rift Valley fever virus in clinical specimens. Journal of Clinical Microbiology 47, 645-651.
Leathwick, D.M. 2004. Managing drench resistance in sheep parasites. Proceedings of the Society of Sheep and Beef Cattle Veterinarians of the New Zealand Veterinary Association, Annual Seminar Jan 2004. 73-81.
Leathwick, D.M., Hosking, B.C., Bisset, S.A., McKay, C.H., 2009. Managing anthelmintic resistance: Is it feasible in New Zealand to delay the emergence of resistance to a new anthelmintic class? New Zealand Veterinary Journal 57, 181-192.
Leathwick, D.M., Miller, C.M., Atkinson, D.S., Haack, N.A., Alexander, R.A., Oliver, A.M., Waghorn, T.S., Potter, J.F., Sutherland, I.A., 2006. Drenching adult ewes: implications of anthelmintic treatments pre- and post-lambing on the development of anthelmintic resistance. New Zealand Veterinary Journal 54, 297-304.
Leathwick, D.M., Miller, C.M., Atkinson, D.S., Haack, N.A., Waghorn, T.S., Oliver, A.M., 2008. Managing anthelmintic resistance: Untreated adult ewes as a source of unselected parasites, and their role in reducing parasite populations. New Zealand Veterinary Journal 56, 184-195.
Leathwick, D.M., Miller, C.M., Brown, A.E., Sutherland, I.A., 1999. The establishment rate of Ostertagia circumcincta and Trichostrongylus colubriformis in lactating Romney ewes. International Journal of Parasitology 29, 315-320.
Leathwick, D.M., Miller, C.M., Fraser, K., 2015. Selection for anthelmintic resistant Teladorsagia circumcincta in pre-weaned lambs by treating their dams with long-acting moxidectin injection. International Journal of Parasitology Drugs Drug Resist 5, 209-214.
Lee, D.L., Martin, J., 1980. The Structure of the Intestine of Nematodirus-Battus and Changes during the Course of an Infection in Lambs. Parasitology 81, 27-&.
Lila, T., Renau, T.E., Wilson, L., Philips, J., Natsoulis, G., Cope, M.J., Watkins, W.J., Buysse, J., 2003. Molecular basis for fungal selectivity of novel antimitotic compounds. Antimicrobial Agents and Chemotherapy 47, 2273-2282.
Little, P.R., Hodges, A., Watson, T.G., Seed, J.A., Maeder, S.J., 2010. Field efficacy and safety of an oral formulation of the novel combination anthelmintic, derquantel-abamectin, in sheep in New Zealand. New Zealand Veterinary Journal 58, 121-129.
Lorenz, T.C., 2012. Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. Journal of Visualised Experiments, e3998.
Macchi, C., Pomroy, W.E., Morris, R.S., Pfeiffer, D.U., West, D.M., 2001. Consequences of anthelmintic resistance on liveweight gain of lambs on commercial sheep farms. New Zealand Veterinary Journal 49, 48-53.
Mapes, C.J., Coop, R.L., 1970. The interaction of infections of Haemonchus contortus and Nematodirus battus in lambs. I. The effect of massive infections of Haemonchus on subsequent infections of Nematodirus. Journal of Comparative Pathology 80, 123-136.
Mapes, C.J., Coop, R.L., 1971. Effect of concurrent and terminated infections of Haemonchus contortus on the development and reproductive capacity of Nematodirus battus. Journal of Comparative Pathology 81, 479-492.

Mapes, C.J., Coop, R.L., 1972. The development of single infections of Nematodirus battus in lambs. Parasitology 64, 197-216.
Mapes, C.J., Coop, R.L., 1973. On the relationship between abomasal electrolytes and some population parameters of the nematodes Haemonchus contortus and Nematodirus battus. Parasitology 66, 95-100.
Mapes, C.J., Coop, R.L., Angus, K.W., 1973. The fate of large infective doses of Nematodirus battus in young lambs. International Journal of Parasitology. 3, 339-347.
Marley, C.L., Cook, R., Barrett, J., Keatinge, R., Lampkin, N.H., McBride, S.D., 2003. The effect of dietary forage on the development and survival of helminth parasites in ovine faeces. Veterinary Parasitology 118, 93-107.
Martin, J., Lee, D.L., 1975. Observations on crystals found in the intestine of Nematodirus battus during the development of immunity to this nematode in lambs. Parasitology 72, 75-80.
Martin, J., Lee, D.L., 1976. Observations on Crystals Found in Intestine of Nematodirus-Battus during Development of Immunity to This Nematode in Lambs. Parasitology 72, 75-&.
Martin, J., Lee, D.L., 1980a. Changes in the Structure of the Male Reproductive-System of Nematodirus-Battus during Its Rejection from Lambs. Parasitology 81, 587-&.
Martin, J., Lee, D.L., 1980b. Nematodirus-Battus - Scanning Electron-Microscope Studies of the Duodenal Mucosa of Infected Lambs. Parasitology 81, 573-&.
Martin, P.J., Anderson, N., Jarrett, R.G., 1989. Detecting benzimidazole resistance with faecal egg count reduction tests and *in vitro* assays. Australian Veterinary Journal 66, 236-240.
Marzio, L., Blennerhassett, P., Chiverton, S., Vermillion, D.L., Langer, J., Collins, S.M., 1990. Altered smooth muscle function in worm-free gut regions of Trichinella-infected rats. American Journal of Physiology 259, G306-313.
McKellar, Q.A., Scott, E.W., 1990. The benzimidazole anthelmintic agents – A review. Journal of Veterinary Pharmacology and Therapeutics 13, 223-247.
McKenna, P.B., Allan, C.M., Taylor, M.J., Townsend, K.G., 1995. The prevalence of anthelmintic resistance in ovine case submissions to animal health laboratories in New Zealand in 1993. New Zealand Veterinary Journal 43, 96-98.
McMahon, C., Edgar, H.W.J., Barley, J.P., Hanna, R.E.B., Brennan, G.P., Fairweather, I., 2017. Control of Nematodirus spp. infection by sheep flock owners in Northern Ireland. Irish Veterinary Journal 70.
Mederos, A.E., Ramos, Z., Banchero, G.E., 2014. First report of monepantel Haemonchus contortus resistance on sheep farms in Uruguay. Parasites and Vectors 7, 598.
Meiri, M., Lister, A.M., Higham, T.F., Stewart, J.R., Straus, L.G., Obermaier, H., Gonzalez Morales, M.R., Marin-Arroyo, A.B., Barnes, I., 2013. Late-glacial recolonization and phylogeography of European red deer (Cervus elaphus L.). Molecular Ecology 22, 4711-4722.
Melville, L., Kenyon, F., Javed, S., McElarney, L., Demeler, J., Skuce, P., 2014. Development of a loop-mediated isothermal amplification (LAMP) assay for the sensitive detection of Haemonchus contortus eggs in ovine faecal samples. Veterinary Parasitology 206, 308-312.
Melville, L.A., McBean, D., Fyfe, A., Campbell, S.J., Palarea-Albaladejo, J., Kenyon, F., 2016. Effect of anthelmintic treatment strategy on strongylid nematode species composition in grazing lambs in Scotland. Parasites and Vectors 9, 199.
Met Office, 2018. Data point [online] available at https://www.metoffice.gov.uk/datapoint [accessed 19th November 2018].
Meyers, L.A., Bull, J.J., 2002. Fighting change with change: adaptive variation in an uncertain world. Trends in Ecology & Evolution 17, 551-557.

Middelberg, A., McKenna, P.B., 1983. Oxfendazole resistance in *Nematodirus spathiger*. New Zealand Veterinary Journal 31, 65-66.
Miller, C.M., Waghorn, T.S., Leathwick, D.M., Candy, P.M., Oliver, A.M.B., Watson, T.G., 2012. The production cost of anthelmintic resistance in lambs. Veterinary Parasitology 186, 376-381.
Minnucci, G., Amicarelli, G., Salmoiraghi, S., Spinelli, O., Guinea Montalvo, M.L., Giussani, U., Adlerstein, D., Rambaldi, A., 2012. A novel, highly sensitive and rapid allele-specific loop-mediated amplification assay for the detection of the JAK2V617F mutation in chronic myeloproliferative neoplasms. Haematologica 97, 1394-1400.
Mitani, Y., Lezhava, A., Kawai, Y., Kikuchi, T., Oguchi-Katayama, A., Kogo, Y., Itoh, M., Miyagi, T., Takakura, H., Hoshi, K., Kato, C., Arakawa, T., Shibata, K., Fukui, K., Masui, R., Kuramitsu, S., Kiyotani, K., Chalk, A., Tsunekawa, K., Murakami, M., Kamataki, T., Oka, T., Shimada, H., Cizdziel, P.E., Hayashizaki, Y., 2007. Rapid SNP diagnostics using asymmetric isothermal amplification and a new mismatch-suppression technology. Nature Methods 4, 257-262.
Mitchell, S., Mearns, R., Richards, I., Donnan, A.A., Bartley, D.J., 2011. Benzimidazole resistance in Nematodirus battus. Veterinary Record. 168, 623-624.
Mohamed, E.K., Al-Farwachi, M.I., 2008. Evaluation of the effect of albendazole against nematodes in sheep in Mosul, Iraq. Iraqi Journal of Veterinary Science 22, 5-7.
Morgan, E.R., Hosking, B.C., Burston, S., Carder, K.M., Hyslop, A.C., Pritchard, L.J., Whitmarsh, A.K., Coles, G.C., 2012. A survey of helminth control practices on sheep farms in Great Britain and Ireland. Veterinary Journal 192, 390-397.
Mori, Y., Hirano, T., Notomi, T., 2006. Sequence specific visual detection of LAMP reactions by addition of cationic polymers. BMC Biotechnology 6, 3.
Mori, Y., Nagamine, K., Tomita, N., Notomi, T., 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochemical and Biophysical Research Communications 289, 150-154.
Morley, F., Bennett, D., McKinney, G., 1969. The effect of intensity of rotational grazing with breeding ewes on phalaris-subterranean clover pastures. Australian Journal of Experimental Agriculture 9, 74-84.
Morley, F.H.W., Donald, A.D., 1980. Farm-Management and Systems of Helminth Control. Veterinary Parasitology 6, 105-134.
Morrison, A.A., Mitchell, S., Mearns, R., Richards, I., Matthews, J.B., Bartley, D.J., 2014. Phenotypic and genotypic analysis of benzimidazole resistance in the ovine parasite Nematodirus battus. Veterinary Research 45, 116.
Mulholland, C., Hoffmann, B., McMenamy, M.J., Korthase, C., Earley, B., Markey, B., Cassidy, J.P., McKillen, J., Allan, G., Welsh, M.D., 2014. The development of an accelerated reverse-transcription loop mediated isothermal amplification for the serotype specific detection of bluetongue virus 8 in clinical samples. Journal of Virological Methods 202, 95-100.
NADIS 2018. Parasite Forecast [online] available at http://www.nadis.org.uk/parasite-forecast/ [accessed 6th December 2018].
Nadler, S.A., Hoberg, E.P., Hudspeth, D.S.S., Rickard, L.G., 2000. Relationships of Nematodirus species and Nematodirus battus isolates (Nematoda: Trichostrongyloidea) based on nuclear ribosomal DNA sequences. Journal of Parasitology 86, 588-601.
Nagamine, K., Hase, T., Notomi, T., 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Molecular and Cellular Probes 16, 223-229.

Nieuwhof, G.J., Bishop, S.C., 2005. Costs of the major endemic diseases of sheep in Great Britain and the potential benefits of reduction in disease impact. Animal Science 81, 23-29.
Niezen, J.H., Robertson, H.A., Sidey, A., Wilson, S.R., 2002a. The effect of pasture species on parasitism and performance of lambs grazing one of three grass-white clover pasture swards. Veterinary Parasitology 105, 303-315.
Niezen, J.H., Waghorn, G.C., Graham, T., Carter, J.L., Leathwick, D.M., 2002b. The effect of diet fed to lambs on subsequent development of Trichostrongylus colubriformis larvae in vitro and on pasture. Veterinary Parasitology 105, 269-283.
Nilyanimit, P., Chansaenroj, J., Poomipak, W., Praianantathavorn, K., Payungporn, S., Poovorawan, Y., 2018. Comparison of Four Human Papillomavirus Genotyping Methods: Next-generation Sequencing, INNO-LiPA, Electrochemical DNA Chip, and Nested-PCR. Annals of Laboratory Medicine 38, 139-146.
Njiru, Z.K., Mikosza, A.S., Armstrong, T., Enyaru, J.C., Ndung'u, J.M., Thompson, A.R., 2008. Loop-mediated isothermal amplification (LAMP) method for rapid detection of Trypanosoma brucei rhodesiense. PLoS Negleted Tropical Diseases 2, e147.
Nolan, T., Connolly, J., Wachendorf, M., 2001. Mixed grazing and climatic determinants of white clover (Trifolium repens L.) content in a permanent pasture. Annals of Botany London 88, 713-724.
Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research 28, e63.
Obendorf, D.L., Nicholls, J., Koen, T., Lacy, E., 1991. Benzimidazole resistant Nematodirus species in Tasmania. Australian Veterinary Journal 68, 72-73.
Obendorf, D.L., Parsons, J., Nicholls, J., 1986. An egg development test for the evaluation of benzimidazole resistance in Nematodirus spathiger. Australian Veterinary Journal. 63, 382-383.
Odoi, A., Gathuma, J.M., Gachuiri, C.K., Omore, A., 2007. Risk factors of gastrointestinal nematode parasite infections in small ruminants kept in smallholder mixed farms in Kenya. BMC Veterinary Research 3, 6.
Okayama, H., Curiel, D.T., Brantly, M.L., Holmes, M.D., Crystal, R.G., 1989. Rapid, nonradioactive detection of mutations in the human genome by allele-specific amplification. Journal of Laboratory and Clinical Medicine 114, 105-113.
Oliver, A., Pomroy, W.E., Leathwick, D.M., 2016a. Benzimidazole resistance in Nematodirus spathiger and N. filicollis in New Zealand. New Zealand Veterinary Journal 64, 201-206.
Oliver, A.M., Leathwick, D.M., Pomroy, W.E., 2014. A survey of the prevalence of Nematodirus spathiger and N. filicollis on farms in the North and South Islands of New Zealand. New Zealand Veterinary Journal 62, 286-289.
Oliver, A.M., Pomroy, W.E., Ganesh, S., Leathwick, D.M., 2016b. Chilling requirements for hatching of a New Zealand isolate of Nematodirus filicollis. Veterinary Parasitology 226, 17-21.
Oloniniyi, O.K., Kurosaki, Y., Miyamoto, H., Takada, A., Yasuda, J., 2017. Rapid detection of all known ebolavirus species by reverse transcription-loop-mediated isothermal amplification (RT-LAMP). Journal of Virological Methods 246, 8-14.
Oscorbin, I.P., Belousova, E.A., Zakabunin, A.I., Boyarskikh, U.A., Filipenko, M.L., 2016. Comparison of fluorescent intercalating dyes for quantitative loop-mediated isothermal amplification (qLAMP). Biotechniques 61, 20-25.
Parida, M., Posadas, G., Inoue, S., Hasebe, F., Morita, K., 2004. Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus. Journal of Clinical Microbiology 42, 257-263.

Parkin, J.T., 1975. Effect of Moisture Stress Upon Hatching of Nematodirus-Battus Larvae. Parasitology 70, 149-155.
Pato, F.J., Vazquez, L., Diez-Banos, N., Lopez, C., Sanchez-Andrade, R., Fernandez, G., Diez-Banos, P., Panadero, R., Diaz, P., Morrondo, P., 2013. Gastrointestinal nematode infections in roe deer (Capreolus capreolus) from the NW of the Iberian Peninsula: assessment of some risk factors. Veterinary Parasitology 196, 136-142.
Poon, L.L., Leung, C.S., Tashiro, M., Chan, K.H., Wong, B.W., Yuen, K.Y., Guan, Y., Peiris, J.S., 2004. Rapid detection of the severe acute respiratory syndrome (SARS) coronavirus by a loop-mediated isothermal amplification assay. Clinical Chemistry 50, 1050-1052.
Prichard, R.K., 1973. The fumarate reductase reaction of Haemonchus contortus and the mode of action of some anthelmintics. International Journal of Parasitology 3, 409-417.
Prichard, R.K., Hennessy, D.R., 1981. Effect of oesophageal groove closure on the pharmacokinetic behaviour and efficacy of oxfendazole in sheep. Research in Veterinary Science 30, 22-27.
Putman, R.J., Moore, N.P., 1998. Impact of deer in lowland Britain on agriculture, forestry and conservation habitats. Mammal Review 28, 141-163.
Ramunke, S., Melville, L., Rinaldi, L., Hertzberg, H., de Waal, T., von Samson-Himmelstjerna, G., Cringoli, G., Mavrot, F., Skuce, P., Krucken, J., Demeler, J., 2016. Benzimidazole resistance survey for Haemonchus, Teladorsagia and Trichostrongylus in three European countries using pyrosequencing including the development of new assays for Trichostrongylus. International Journal of Parasitology: Drugs and Drug Resistance 6, 230-240.
Rashwan, N., Bourguinat, C., Keller, K., Gunawardena, N.K., de Silva, N., Prichard, R., 2016. Isothermal Diagnostic Assays for Monitoring Single Nucleotide Polymorphisms in Necator americanus Associa\ ted with Benzimidazole Drug Resistance. PLoS Neglected Tropical Diseases 10, e0005113.
Rattray, P.V., Joyce, J.P., 1974. Nutritive value of white clover and perennial ryegrass .4. Utilization of dietary energy. New Zealand Journal of Agricultural Research 17, 401-406.
Redman, E., Whitelaw, F., Tait, A., Burgess, C., Bartley, Y., Skuce, P.J., Jackson, F., Gilleard, J.S., 2015. The emergence of resistance to the benzimidazole anthlemintics in parasitic nematodes of livestock is characterised by multiple independent hard and soft selective sweeps. PLoS Neglected Tropical Diseases 9, e0003494.
Rickard, L.G., Hoberg, E.P., Bishop, J.K., Zimmerman, G.L., 1989. Epizootiology of Nematodirus-Battus, Nematodirus-Filicollis, and Nematodirus-Spathiger (Nematoda, Trichostrongyloidea) in Western Oregon. Proceedings of the Helminthological Society of Washington 56, 104-115.
Rodgers, J.L., 1983. Change in N battus epidemiology. The Veterinary Record 112, 261-262.
Rodriguez-Vivas, R., Perez-Cogollo, L., Trinidad-Martinez, I., Ojeda-Chi, M., Gonzalez-Santana, M., 2017. First report of Nematodirus filicollis natural infection in a sheep from the mexican sub-humid tropics. Rev Mvz Cordoba 22, 6256-6265.
Rogers, W.P., 1958. Physiology of the hatching of eggs of Ascaris lumbricoides. Nature 181, 1410-1411.
Rosalinski-Moraes, F., Moretto, L.H., Bresolin, W.S., Gabrielli, I., Kafer, L., Zanchet, I.K., Sonaglio, F., Thomas-Socool, V., 2007. Resistencia anti-helmintica em rebanhos ovinos da regiao da associacao dos municipios do Alto Irani (Amai), oeste de Santa Catarina. Ciencia Animal Brasileira 8, 559 - 565.

Rose, H., Wang, T., van Dijk, J., Morgan, E.R., 2015. GLOWORM-FL: A simulation model of the effects of climate and climate change on the free-living stages of gastro-intestinal nematode parasites of ruminants. Ecological Modelling 297, 232-245.
Rothwell, J.T., Sangster, N.C., 1993. An in vitro assay utilising parasitic larval Haemonchus contortus to detect resistance to closantel and other anthelmintics. International journal for parasitology 23, 573-578.
Roush, R.T., McKenzie, J.A., 1987. Ecological Genetics of Insecticide and Acaricide Resistance. Annual Review of Entomology 32, 361-380.
Rowlands, D.A., Probert, A.J., 1972. Some Pathological Changes in Young Lambs Experimentally Infected with Nematodirus battus. Research in Veterinary Science 13, 323-&.
Sangster, N.C., Whitlock, H.V., Russ, I.G., Gunawan, M., Griffin, D.L., Kelly, J.D., 1979. Trichostrongylus colubriformis and Ostertagia circumcincta resistant to levamisole, morantel tartrate and thiabendazole: occurrence of field strains. Research in Veterinary Science 27, 106-110.
Santos, J., Vasconcelos, J.F., Frota, G.A., Ribeiro, W.L.C., Andre, W.P.P., Vieira, L.D.S., Teixeira, M., Bevilaqua, C.M.L., Monteiro, J.P., 2017. Haemonchus contortus beta-tubulin isotype 1 gene F200Y and F167Y SNPs are both selected by ivermectin and oxfendazole treatments with differing impacts on anthelmintic resistance. Veterinary Parasitology 248, 90-95.
Sargison, N.D., Wilson, D.J., Scott, P.R., 2012. Observations on the epidemiology of autumn nematodirosis in weaned lambs in a Scottish sheep flock. Veterinary Record 170, 391-347.
Scott, E.W., Mitchell, E.S., Armour, J., Bairden, K., Soutar, A., Bogan, J.A., 1989. Level of benzimidazole resistance in a strain of Ostertagia circumcincta studied over several infections in lambs. Veterinary Parasitology 30, 305-314.
Scott, I., Pomroy, W.E., Kenyon, P.R., Smith, G., Adlington, B., Moss, A., 2013. Lack of efficacy of monepantel against Teladorsagia circumcincta and Trichostrongylus colubriformis. Veterinary Parasitology 198, 166-171.
Silvestre, A., Cabaret, J., 2002. Mutation in position 167 of isotype 1 beta-tubulin gene of Trichostrongylid nematodes: role in benzimidazole resistance? Molecular and Biochemical Parasitology 120, 297-300.
Silvestre, A., Humbert, J.F., 2002. Diversity of benzimidazole-resistance alleles in populations of small ruminant parasites. International Journal for Parasitology 32, 921-928.
Silvestre, A., Sauve, C., Cortet, J., Cabaret, J., 2009. Contrasting genetic structures of two parasitic nematodes, determined on the basis of neutral microsatellite markers and selected anthelmintic resistance markers. Molecular Ecology 18, 5086-5100.
Simsek, M., Adnan, H., 2000. Effect of single mismatches at 3'-end of primers on polymerase chain reaction. Journal of Research in the Medical Sciences 2, 11-14.
Skuce, P., Stenhouse, L., Jackson, F., Hypsa, V., Gilleard, J., 2010. Benzimidazole resistance allele haplotype diversity in United Kingdom isolates of Teladorsagia circumcincta supports a hypothesis of multiple origins of resistance by recurrent mutation. International Journal for Parasitology 40, 1247-1255.
Smeal, M.G., Gough, P.A., Jackson, A.R., Hotson, I.K., 1968. The occurrence of strains of Haemonchus contortus resistant to thiabendazole. Australian Veterinary Journal 44, 108-109.
Smith, H.J., Hines, J.G., 1987. Nematodirus battus in Canadian Sheep. Canadian Veterinary Journal 28, 256.

Soleimani, M., Shams, S., Majidzadeh, A.K., 2013. Developing a real-time quantitative loop-mediated isothermal amplification assay as a rapid and accurate method for detection of Brucellosis. Journal of Applied Microbiology 115, 828-834.
Stamp, J.T., Dunn, A.M., 1955. Lamb Losses from Worms. *Nematodirus* Infestation. Scottish Agriculture 34, 213-215.
Stanton, D.W., Mulville, J.A., Bruford, M.W., 2016. Colonization of the Scottish islands via long-distance Neolithic transport of red deer (Cervus elaphus). Proceedings of The Royal Society of Biological sciences 283.
Stubbings, L.A. 2018. SCOPS Nematodirus forecast [online] available at http://www.scops.org.uk/forecasts/nematodirus-forecast/ [accessed 15th December 2018].
Suarez, V.H., Cristel, S.L., 2014. Risk factors for anthelmintic resistance development in cattle gastrointestinal nematodes in Argentina. Revista Brasileira Parasitologia Veterinaria 23, 129-135.
Suter, R.J., Besier, R.B., Perkins, N.R., Robertson, I.D., Chapman, H.M., 2004. Sheep-farm risk factors for ivermectin resistance in Ostertagia circumcincta in Western Australia. Preventive Veterinary Medicine 63, 257-269.
Taylor, D.M., Thomas, R.J., 1986. The development of immunity to Nematodirus battus in lambs. International Journal of Parasitology 16, 43-46.
Taylor, M.A., Hunt, K.R., 1988. Field observations on the control of ovine parasitic gastroenteritis in south-east England. Veterinary Record 123, 241-245.
Taylor, M.A., Hunt, K.R., 1989. Aanthelmintic drug resistance in the UK. Veterinary Record 125, 143-147.
Taylor, S.M., Mallon, T.R., Blanchflower, W.J., Kennedy, D.G., Green, W.P., 1992. Effects of diet on plasma concentrations of oral anthelmintics for cattle and sheep. Veterinary Record 130, 264-268.
Teagasc, 2014. Pocket mannual for reseeding [online] available at https://www.teagasc.ie/media/website/animals/beef/pocketmanualforreseeding.pdf [accessed 12th December 2018].
Terefe, G., Lacroux, C., Andreoletti, O., Grisez, C., Prevot, F., Bergeaud, J.P., Penicaud, J., Rouillon, V., Gruner, L., Brunel, J.C., Francois, D., Bouix, J., Dorchies, P., Jacquiet, P., 2007. Immune response to Haemonchus contortus infection in susceptible (INRA 401) and resistant (Barbados Black Belly) breeds of lambs. Parasite Immunology 29, 415-424.
Thomas, D.R., 1990. The epidemiology of *Nematodirus battus* - Is it changing? Parasitology 102, 147-155.
Thomas, E., Morgan, E., Paton, N., 2015. WAARD Project: Wales Against Anthelmintic Resistance Development [online] available at https://hccmpw.org.uk/images/resources/WAARD_FINAL_PROJECT_REPORT_1_-_19-11-15.pdf [accessed 15th December 2018].
Thomas, R.J., 1959a. A comparative study of the life histories of Nematodirus battus and N. filicollis, nematode parasites of sheep. Parasitology 49, 374-386.
Thomas, R.J., 1959b. Field studies on the seasonal incidence of Nematodirus battus and N. filicollis in sheep. Parasitology 49, 387-410.
Thomas, R.J., Stevens, A.J., 1956. Some Observations on Nematodirus Disease in Northumberland and Durham. Veterinary Record 68, 471-475.
Thomas, R.J., Stevens, A.J., 1960. Ecological studies on the development of the pasture stages of Nematodirus battus and N. filicollis, nematode parasites of sheep. Parasitology 50, 31-49.

Thorburn, F., Bennett, S., Modha, S., Murdoch, D., Gunson, R., Murcia, P.R., 2015. The use of next generation sequencing in the diagnosis and typing of respiratory infections. Journal of Clinical Virology 69, 96-100.
Torina, A., Dara, S., Marino, A.M.F., Sparagano, O.A.E., Vitale, F., Reale, S., Caracappa, S., 2004. Study of gastrointestinal nematodes in sicilian sheep and goats. Annals of the New York academy of science 1026, 187-194.
Trinh, T.N.D., Lee, N.Y., 2018. A rapid and eco-friendly isothermal amplification microdevice for multiplex detection of foodborne pathogens. Lab on a chip 18, 2369-2377.
Tsai, S.M., Chan, K.W., Hsu, W.L., Chang, T.J., Wong, M.L., Wang, C.Y., 2009. Development of a loop-mediated isothermal amplification for rapid detection of orf virus. Journal of Virological Methods 157, 200-204.
Vadlejch, J., Kopecky, O., Kudrnacova, M., Cadkova, Z., Jankovska, I., Langrova, I., 2014. The effect of risk factors of sheep flock management practices on the development of anthelmintic resistance in the Czech Republic. Small Ruminant Research 117, 183-190.
van den Brom, R., Moll, L., Kappert, C., Vellema, P., 2015. Haemonchus contortus resistance to monepantel in sheep. Veterinary Parasitology 209, 278-280.
van Dijk, J., David, G.P., Baird, G., Morgan, E.R., 2008. Back to the future: developing hypotheses on the effects of climate change on ovine parasitic gastroenteritis from historical data. Veterinary Parasitology 158, 73-84.
van Dijk, J., de Louw, M.D., Kalis, L.P., Morgan, E.R., 2009. Ultraviolet light increases mortality of nematode larvae and can explain patterns of larval availability at pasture. International Journal of Parasitology 39, 1151-1156.
van Dijk, J., Morgan, E.R., 2008. The influence of temperature on the development, hatching and survival of Nematodirus battus larvae. Parasitology 135, 269-283.
van Dijk, J., Morgan, E.R., 2009. Hatching behaviour of Nematodirus filicollis in a flock co-infected with Nematodirus battus. Parasitology 136, 805-811.
van Dijk, J., Morgan, E.R., 2010. Variation in the hatching behaviour of Nematodirus battus: polymorphic bet hedging? International Journal of Parasitology 40, 675-681.
van Dijk, J., Morgan, E.R., 2012. The influence of water and humidity on the hatching of Nematodirus battus eggs. Journal of Helminthology 86, 287-292.
van Houtert, M.F.J., Barger, I.A., Steel, J.W., 1995. Dietary protein for young grazing sheep - Interactions with gastrointestinal parasitism. Veterinary Parasitology 60, 283-295.
van Wyk, J.A., 2001. Refugia - overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. Onderstepoort Journal of Veterinary Research 68, 55-67.
van Wyk, J.A., Malan, F.S., 1988. Resistance of field strains of Haemonchus contortus to ivermectin, closantel, rafoxanide and the benzimidazoles in South Africa. Veterinary Record 123, 226-228.
Veigas, B., Pedrosa, P., Couto, I., Viveiros, M., Baptista, P.V., 2013. Isothermal DNA amplification coupled to Aunanoprobes for detection of mutations associated to Rifampicin resistance in Mycobacterium tuberculosis. Journal of Nanobiotechnology 11.
Verschave, S.H., Charlier, J., Rose, H., Claerebout, E., Morgan, E.R., 2016. Cattle and Nematodes Under Global Change: Transmission Models as an Ally. Trends Parasitology 32, 724-738.
Vineer, H.R., Steiner, T., Knapp-Lawitzke, F., Bull, K., von Son-de Fernex, E., Bosco, A., Hertzberg, H., Demeler, J., Rinaldi, L., Morrison, A.A., Skuce, P., Bartley, D.J., Morgan, E.R., 2016. Implications of between-isolate variation for climate change impact modelling of Haemonchus contortus populations. Veterinary Parasitology 229, 144-149.

Vlassoff, A., Kettle, P.R., 1985. Register of anthelmintic resistant nematodes in New Zealand. New Zealand Veterinary Journal 33, 71-71.
von Samson-Himmelstjerna, G., von, W.C., Sievers, G., Schnieder, T., 2002. Comparative use of faecal egg count reduction test, egg hatch assay and beta-tubulin codon 200 genotyping in small strongyles (cyathostominae) before and after benzimidazole treatment. Veterinary Parasitology. 108, 227-235.
von Samson-Himmelstjerna, G., Walsh, T.K., Donnan, A.A., Carriere, S., Jackson, F., Skuce, P.J., Rohn, K., Wolstenholme, A.J., 2009. Molecular detection of benzimidazole resistance in Haemonchus contortus using real-time PCR and pyrosequencing. Parasitology, 1-10.
Waghorn, T.S., Miller, C.M., Oliver, A.M., Leathwick, D.M., 2009. Drench-and-shift is a high-risk practice in the absence of refugia. New Zealand Veterinary Journal 57, 359-363.
Wallace, D.I., Southworth, B.S., Shi, X., Chipman, J.W., Githeko, A.K., 2014. A comparison of five malaria transmission models: benchmark tests and implications for disease control. Malaria Journal 13, 268.
Wallace, D.S., Bairden, K., Duncan, J.L., Fishwick, G., Gill, M., Holmes, P.H., McKellar, Q.A., Murray, M., Parkins, J.J., Stear, M.J., 1995. Influence of supplementation with dietary soyabean meal on resistance to haemonchosis in Hampshire down lambs. Research in Veterinary Science 58, 232-237.
Waller, P.J., Donald, A.D., Dobson, R.J., Lacey, E., Hennessy, D.R., Allerton, G.R., Prichard, R.K., 1989. Changes in anthelmintic resistance status of Haemonchus contortus and Trichostrongylus colubriformis exposed to different anthelmintic selection pressures in grazing sheep. International Journal for parasitology 19, 99-110.
Waters, R.A., Fowler, V.L., Armson, B., Nelson, N., Gloster, J., Paton, D.J., King, D.P., 2014. Preliminary validation of direct detection of foot-and-mouth disease virus within clinical samples using reverse transcription loop-mediated isothermal amplification coupled with a simple lateral flow device for detection. PLoS One 9, e105630.
Whittaker, J.H., Carlson, S.A., Jones, D.E., Brewer, M.T., 2017. Molecular mechanisms for anthelmintic resistance in strongyle nematode parasites of veterinary importance. Journal of Veterinary Pharmacology Ther 40, 105-115.
Winter, M.D., Wright, C., Lee, D.L., 1997a. The effect of dexamethasone on resistance of older lambs to infection with Nematodirus battus. Journal of Helminthology 71, 133-138.
Winter, M.D., Wright, C., Lee, D.L., 1997b. The mast cell and eosinophil response of young lambs to a primary infection with Nematodirus battus. Parasitology 114 (Pt 2), 189-193.
Winter, M.D., Wright, C., Wakelin, D., Lee, D.L., 1996. The serum immune response of young lambs to a primary infection with Nematodirus battus. Parasitology 113 (Pt 5), 491-496.
Xu, C.P., Wang, H.L., Jin, H.L., Feng, N., Zheng, X.X., Cao, Z.G., Li, L., Wang, J.Z., Yan, F.H., Wang, L.N., Chi, H., Gai, W.W., Wang, C., Zhao, Y.K., Feng, Y., Wang, T.C., Gao, Y.W., Lu, Y.Y., Yang, S.T., Xia, X.Z., 2016. Visual detection of Ebola virus using reverse transcription loop-mediated isothermal amplification combined with nucleic acid strip detection. Archives of Virology 161, 1125-1133.
Yatabe, T., Arriagada, G., Hamilton-West, C., Urcelay, S., 2011. Risk factor analysis for sea lice, Caligus rogercresseyi, levels in farmed salmonids in southern Chile. Journal of Fish Diseases 34, 345-354.
Yitshak-Sade, M., Kloog, I., Novack, V., 2017. Do air pollution and neighborhood greenness exposures improve the predicted cardiovascular risk? Environment International 107, 147-153.

Yongkiettrakul, S., Kampeera, J., Chareanchim, W., Rattanajak, R., Pornthanakasem, W., Kiatpathomchai, W., Kongkasuriyachai, D., 2017. Simple detection of single nucleotide polymorphism in Plasmodium falciparum by SNP-LAMP assay combined with lateral flow dipstick. Parasitology International 66, 964-971.

Yu, F., Wen, Y., Wang, J., Gong, Y., Feng, K., Ye, R., Jiang, Y., Zhao, Q., Pan, P., Wu, H., Duan, S., Su, B., Qiu, M., 2018. The Transmission and Evolution of HIV-1 Quasispecies within One Couple: a Follow-up Study based on Next-Generation Sequencing. Nature Science Reports 8, e1404.

Zanoli, L.M., Spoto, G., 2013. Isothermal amplification methods for the detection of nucleic acids in microfluidic devices. Biosensors (Basel) 3, 18-43.

Zayed, Y. 2016. Agriculture: historical statistics [online] available at https://researchbriefings.parliament.uk/ResearchBriefing/Summary/SN03339 [accessed 15th December 2018].

Zhang, C., Yao, Y., Zhu, J.L., Zhang, S.N., Zhang, S.S., Wei, H., Hui, W.L., Cui, Y.L., 2016. Establishment and application of a real-time loop-mediated isothermal amplification system for the detection of CYP2C19 polymorphisms. Nature Science Reports 6, e26533.

Zhang, C., Zhu, J.L., Yang, J.C., Wan, Y.S., Ma, T., Cui, Y.L., 2015. Determination of ABO blood group genotypes using the real-time loop-mediated isothermal amplification method. Molecular Medicine Reports 12, 5963-5966.